The Biological Activity of *Bacteroides* Surface Polysaccharides

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Contents

Abbreviations

Abbreviations frequently used and/or novel to this thesis are listed below.

Ab	antibody
Ag	antigen
ATCC	american type culture collection
BPI	bactericidal/permeability increasing protein
CH_{50}	haemolytic complement value
CHO	carbohydrate
cfu	colony forming units
CP	capsular polysaccharide
DIC	disseminated intravascular coagulation
ECV	extracellular vesicles
EDL	electron dense layer
ELISA	enzyme-linked immunosorbent assay
EPS	extracellular polysaccharide
FCS	foetal calf serum
HDL	high density lipoprotein
HISS	heat inactivated sheep serum
IL	interleukin
KDO	3-deoxy-D-manno-octulosonic acid
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MNL	mononuclear leukocytes
MOF	multi-organ failure
MPRL	departmental stock culture
mwt	molecular weight
NB	nutrient broth
NCTC	national type culture collection
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCP	phenol-chloroform-petroleum extraction method
PF	pyrogen free
PMN	polymorphonuclear neutrophils
PPY	proteose peptone yeast extract broth
PW	phenol-water extraction method
SIRS	systemic inflammatory response syndrome
sTNFr	soluble tumour necrosis factor receptor
TNF	tumour necrosis factor
Triton	triton-Mg^{2+} extraction method
VT	Van Tassell & Wilkins minimal defined media

Abstract

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, is implicated as the key factor in the development of the Systemic Inflammatory Response Syndrome (SIRS). LPS can arise from an underlying bacteraemia, but given that the majority of patients with SIRS have no detectable bacteraemia, then LPS derived from the gut must be considered. *Bacteroides* species outnumber the enterobacteria such as *E. coli* in the gut by approximately 1000-fold. Although *Bacteroides* LPS is less endotoxic, by simple arithmetic there must be as much biological potential from the LPS of *Bacteroides* as from *E. coli.* This thesis re-examines the biological activity of *Bacteroides* LPS and its possible role in the development of SIRS.

LPSs were extracted from seven *Bacteroides* species by three different techniques: the phenol-water (PW), the phenol-chloroform-petroleum (PCP) and Triton-Mg^{2+}. The biological activity of these *Bacteroides* LPSs was compared to that of an *E. coli* O18K$^-$ LPS control. In general, *Bacteroides* LPSs prepared by the PW method were found to have a significantly higher activity in a mouse lethality model, LAL assay, TNF and IL-8 induction assays, than LPS extracted by the PCP or Triton methods. *Bacteroides* LPS extracted by the PCP method had consistently low activity in all assays. LPS from *B. fragilis* NCTC 9343 and *B. caccae* had a consistently higher activity than LPS from *B. vulgatus* and *B. thetaiotaomicron* in most assays. Differences in activity between *B. fragilis* NCTC 9343 LPS grown in different media was seen. The PW method selected for greater amounts of carbohydrate and KDO and the PCP the least. Further information from sub-population studies, Percoll profiles, chemotype on PAGE and chemical analysis failed to account for differences in biological activity between extraction methods and *Bacteroides* species.

Comparing PW-LPS from *Bacteroides* with that from *E. coli*, cytokine induction was examined from three different cell populations: human mononuclear leukocytes (MNL), THP-1 cells (with and without enhancement for CD14 by vitamin D_3) and peritoneal macrophages from C3H/HeJ (LPS-non-responder) and C3H/HeN (LPS-responder) mice. In brief, *E. coli* O18K$^-$ LPS produced multiple peaks of TNF production through a CD14 dependent pathway, while *B. fragilis* NCTC 9343 LPS could only produce one peak of TNF through a pathway independent of CD14. In contrast, *E. coli* LPS induced a single peak of IL-8, whereas *Bacteroides* LPS induced two peaks. Both LPSs induced IL-8 through a CD14 dependent pathway. In IL-6 production, both *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 LPS induced a single peak through a pathway independent of CD14. Cytokine induction by capsular polysaccharide (CP) was also examined. Most activity was seen in the low mwt CP (which contained more carbohydrate and KDO) but on a scale much lower than LPS. An excess of *Bacteroides* LPS (extracted by all three methods) or CP masked the effect of *E. coli* LPS in producing the cytokines TNF, IL-8 and IL-6. Several anti-*Bacteroides* mAbs inhibited TNF production by both *E. coli* and *B. fragilis* NCTC 9343 LPS but did not inhibit IL-8 production.

Finally, studies into the mitogenicity of *Bacteroides* LPS and CP indicate that *B. vulgatus* and high mwt CP were more mitogenic than *B. fragilis* NCTC 9343 LPS in mixed spleen cells, B cells and T cells from LPS-responder mice.

Summary

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, is implicated as the key factor in the development of the Systemic Inflammatory Response Syndrome (SIRS). LPS can arise from an underlying bacteraemia, but given that the majority of patients with SIRS have no detectable bacteraemia, then LPS derived from the gut must be considered. *Bacteroides* species outnumber the enterobacteria such as *E. coli* in the gut by approximately 1000-fold. *B. fragilis* is the most common anaerobe in clinical infections such as intra-abdominal abscesses. Although *Bacteroides* LPS is less endotoxic, by simple arithmetic there must be as much biological potential from the LPS of *Bacteroides* as from *E. coli.*

This thesis re-examines the biological activity of *Bacteroides* LPS and its possible role in the development of SIRS.

LPSs were extracted from seven *Bacteroides* species by three different techniques- the phenol- water (PW), the phenol-chloroform-petroleum (PCP) and Triton-Mg^{2+}. The strains selected included two different *B. fragilis* strains, one of which was grown in two different media. A batch of sodium salt-form LPS was also prepared. The biological activity of these *Bacteroides* LPSs was compared to that of an *E. coli* O18K$^-$ LPS control.

In general, *Bacteroides* LPSs prepared by the PW method were found to have a significantly higher activity in a mouse lethality model, LAL assay and TNF and IL-8 induction assays, than LPS extracted by the PCP or Triton methods. *Bacteroides* LPS extracted by the PCP method had consistently low activity in all assays. LPS from *B. fragilis* NCTC 9343 and *B. caccae* had a consistently higher activity than LPS from

B. vulgatus and *B. thetaiotaomicron* in most assays. In the LAL assay and mouse lethality model, native LPS was approximately two-fold more active than sodium salt-form LPS. There was no clear difference in activity between native and sodium salt-form LPS in the TNF induction assay. Differences in activity between *B. fragilis* NCTC 9343 LPS grown in different media were seen. The PW method selected for greater amounts of carbohydrate and KDO and the PCP the least. Further information from sub-population studies, Percoll profiles, chemotype on PAGE and chemical analysis failed to account for differences in biological activity between extraction methods and *Bacteroides* species.

Comparing PW-LPS from *Bacteroides* with that from *E. coli*, in the mouse lethality model, the *E. coli* LPS was approximately 5000-fold more active than the most active *Bacteroides* LPS. However in the LAL assay, *B. fragilis* NCTC 9343 and *B. caccae* LPS had higher activities (up to 30-fold) than *E. coli* LPS, with the other *Bacteroides* species being up to 15-fold less active than the *E. coli* LPS.

Cytokine induction was examined from three different cell populations:- human mononuclear leukocytes (MNL), THP-1 cells (with and without enhancement for CD14 by vitamin D_3) and peritoneal macrophages from C3H/HeJ (LPS-non-responder) and C3H/HeN (LPS-responder) mice.

The stimulation of TNF production by *E. coli* LPS was between 2 and 4-fold more than *Bacteroides* LPS in MNL, in THP-1 cells (with enhancement for CD14) and in peritoneal macrophages from C3H/HeN mice. In THP-1 cells (without enhancement for CD14) there was no significant difference in TNF production between *E. coli* and *Bacteroides* LPS. In peritoneal macrophages from C3H/HeJ mice *E. coli* LPS stimulated no TNF production, but there was no significant difference in TNF

production from peritoneal macrophages from C3H/HeJ and C3H/HeN mice by *Bacteroides* LPS. In all cell populations, there was a peak of TNF production after approximately 4 h stimulation with all LPSs tested. However, other peaks of TNF production were seen in MNL and THP-1 cells (with enhancement for CD14) after stimulation with *E. coli* LPS only. An anti-CD14 monoclonal antibody (mAb) did not inhibit *Bacteroides* LPS stimulated TNF production. However, *E. coli* LPS stimulated TNF release was inhibited by a CD14 mAb, most noticeably in MNL and THP-1 cells (with enhancement for CD14).

In contrast, *E. coli* LPS induced a single peak of IL-8 at 6 h, whereas *Bacteroides* LPS induced two peaks at 9 and 12 h from human MNL. IL-8 production by both *E. coli* LPS and *Bacteroides* LPS could be slightly inhibited by a CD14 mAb in all cell populations tested. IL-6 production by human MNL was comparable between *E. coli* LPS and *B. fragilis* NCTC 9343 LPS. Both LPSs induced a single peak of IL-6 at 6 h and IL-6 production by both LPSs was not inhibited by a CD14 mAb.

As it was presumed that *Bacteroides* LPS preparations in this thesis may not be pure but heterogeneous with capsular polysaccharide (CP), the ability of CP from *B. fragilis* NCTC 9343 to induced cytokine production was examined. Most cytokine induction activity was seen in the low mwt CP (which contained more carbohydrate and KDO) but on a scale much lower than LPS. In human MNL, CP produced a single peak of TNF at 6 h. Low mwt CP induced one peak of IL-8 at 10 h whereas high mwt CP induced increasing quantities of IL-8 over time with maximum levels observed after 24 h stimulation. An excess of *Bacteroides* LPS (extracted by all three methods) or CP masked the effect of *E. coli* LPS in producing the cytokines TNF, IL-8 and IL-6. Several anti-*Bacteroides* mAbs inhibited TNF production by both *E. coli* and *B. fragilis* NCTC 9343 LPS but did not inhibit IL-8 production.

Studies into the mitogenicity of *Bacteroides* LPS and CP indicate that *B. vulgatus* and high mwt CP were more mitogenic than *B. fragilis* NCTC 9343 LPS in mixed spleen cells, B cells and T cells from LPS-responder mice. However, firm conclusions cannot be drawn from the mitogenicity studies due to lack of flow cytometry evidence that homogeneous cell populations were prepared.

Declaration

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Introduction

1.1 GENERAL INTRODUCTION

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria. It is implicated as the key factor in the development of the Systemic Inflammatory Response Syndrome (SIRS). This is a common generalised condition and the mortality rate still remains high after the onset of shock despite recent advances in antimicrobial therapy and in our understanding of the pathophysiology of SIRS. In approximately 50 per cent (Bone 1993, Gibb 1993) of patients with SIRS the source of LPS is thought to originate from the gut. *Bacteroides* species are the predominant Gram-negative organisms of the gut flora, with *B. fragilis* the most common anaerobic organism isolated from clinical infections. The *Bacteroides* species and their LPS are now reviewed with regards to their possible role in SIRS.

1.2 THE GENUS *BACTEROIDES*

The genus *Bacteroides* recently underwent taxonomic change (Shah & Collins 1989) and was restricted to those species in which the DNA base composition was within the range G + C 30-40 % homology. The genus now contains the following eleven species- *B. fragilis*, *B. vulgatus*, *B. eggerthii*, *B. thetaiotaomicron*, *B. merdae*, *B. caccae*, *B. variabilis*, *B. uniformis*, *B. ovatus*, *B. distasonis* and *B. stercoris*, with other organisms being reassigned to such genera as the *Prevotella* and *Porphyromonas*. They are all non-motile, non-spore forming, Gram-negative rods or cocco-bacilli, bile tolerant and strict anaerobes. They are also all saccharolytic, produce major amounts of acetate and succinate as metabolic end products, they possess enzymes of the hexose monophosphate shunt and the pentose phosphate pathway (Duerden & Drasar 1991, Shah & Collins 1990).

1.3 *BACTEROIDES* AS COMMENSALS

Bacteroides species are normal constituents of the faecal flora. Anaerobes make up approximately 99-99.9% of the faecal bacterial mass, outnumbering the facultative aerobes by a factor of 100-1000. *Bacteroides* species are the predominant organisms in the human intestine accounting for 30% of the species isolated (Duerden & Drasar 1991), being present at a concentration of approximately 10^{11} organisms/g of faeces (Hentges 1993, Finegold 1995).

The most common *Bacteroides* species isolated from faeces is *B. vulgatus*. Namavar and colleagues (1989) estimated that in faeces *B. vulgatus* accounted for 44% of isolates and *B. fragilis* at 4%, accounting for only a minor proportion of isolates. However, in the colonic mucosa *B. fragilis* was found to be the most predominant organism at 44%, compared to 35% of *B. vulgatus*.

The proportion of the organisms relative to other faecal organisms varies greatly with diet. *Bacteroides* species are found in greater numbers in the western diet where bile production is stimulated by food with a high fat content compared to countries where a vegetarian diet is more common (Drasar 1974).

1.4 *BACTEROIDES* AS PATHOGENS

B. fragilis is the anaerobe most frequently isolated from clinical infections such as bacteraemia, brain, liver and lung abscesses and most commonly from intra-abdominal abscesses. One study reported that *B. fragilis* was present in approximately 90% of cases of peritonitis (Tally & Ho 1987) and *Escherichia coli (E. coli)* in approximately 60% of cases. Although the incidence of *B. fragilis* in bacteraemia is higher than other *Bacteroides* species, it has been reported that the relative mortality rates are greater with *B. distasonis*, *B. thetaiotaomicron* and *B. vulgatus* (Brook 1989).

1.5 GENERAL VIRULENCE FACTORS ASSOCIATED WITH *BACTEROIDES* SPECIES

The majority of literature published on the pathogenicity of *Bacteroides* species has concentrated on *B. fragilis* since it is the most frequently associated with clinical infection.

Possible virulence determinants of *B. fragilis* include fimbriae, production of enterotoxins and degradative enzymes, synergy with *E. coli*, capsular polysaccharides and lipopolysaccharides, with the latter three thought to be the most important (Patrick 1993).

1.5.1 Fimbriae

The presence of fimbriae expressed by *B. fragilis* was first reported in 1984 by Pruzzo *et al* who described fimbriae of approximately 30 nm in diameter. They showed that a fimbriated strain adhered better to human epithelial cells than did non-fimbriated strains, suggesting a role for fimbriae in the colonisation of the intestinal tract. Later in 1987, Van Doorn and colleagues described fimbriae of 4-5 nm in diameter. They showed that fimbrial expression was reduced at low iron concentrations and at low temperatures. This observation helps explain why the presence of fimbriae in *Bacteroides* species recovered from abscesses and normal flora was greater than those recovered from the blood in a study by Brook *et al* (1992). Their findings suggest a role for these organisms to adhere to mucous membranes better than their ability to spread systemically.

1.5.2 Enterotoxins

Enterotoxigenic strains of *B. fragilis* have been isolated in a study from 8 out of 44 patients with diarrhoea. As few as 5 x 10^3 cfu of enterotoxigenic *B. fragilis* caused

fatal enteric disease in a rabbit model (Myers *et al* 1987). An enterotoxin from strains of *B. fragilis* has been isolated (Van Tassell *et al* 1992) which has a strong secretory response in the lamb ileal-loop assay as well as a cytotoxic response on HT-29 colon carcinoma cells. Recently, this enterotoxin has been found to be a metalloprotease (Moncrieff *et al* 1995).

1.5.3 Degradative Enzymes

B. fragilis can release extracellular enzymes that can degrade the components of the hosts extracellular matrix such as hyaluronidase and chondroitin sulphatase. They can also produce DNAse, lipases, proteases and neuraminadases which can break down the host cells and tissues (Gibson & MacFarlane 1988, Hofstad 1984). Neuraminadase production is high in clinical isolates of *B. fragilis* (Hofstad 1984). This enzyme cleaves sialic acid from oligosaccharides on host cell glycoproteins and glycolipids. Sialic acid is commonly found on cells of the immune system and therefore released neuraminadases may disrupt the normal function of the immune system (Imai *et al* 1991). Finally, a growing number of *Bacteroides* species have been shown to produce penicillinases which may protect *Bacteroides* strains being eradicated by β-lactam antibiotics (Horn *et al* 1992).

1.5.4 Synergism

B. fragilis species are most often isolated from intra-abdominal abscesses in conjunction with other anaerobes and/or facultative bacteria. Numerous investigators have reported a synergistic relationship between *B. fragilis* and *E. coli* (Tanaka *et al* 1994, Rotstein *et al* 1989, Onderdonk *et al* 1976, Brook 1994, Finlay-Jones *et al* 1991).

It is thought that the aerobic bacteria such as *E. coli* provides nutrients, low pH and oxidation-reduction potential which allows the *B. fragilis* to grow. Intra-abdominal abscesses will not form unless both organisms are present (Onderdonk *et al* 1976).

The ability of *B. fragilis* to inhibit phagocytic killing of *E. coli* by polymorphonuclear neutrophils (PMN) has been well described and appears to be mediated by at least two mechanisms (Rotstein *et al* 1985). *Bacteroides* species are able to deplete both heat stable and heat labile opsonins resulting in impaired opsonisation of their aerobic partner and thus inhibiting phagocytic killing of the aerobe. What component of *Bacteroides* is causing this effect has not been clearly defined. Secondly, short chain fatty acids (SCFA) (mainly succinic, acetic, lactic, formic and fumaric acids) produced by *B. fragilis* during the stationary phase of growth have been shown to cause the impairment of the microbicidal activity of PMNs. The observation that inhibition was maximal at low extracellular pH led to the finding that the SCFA mediated this effect by shuttling protons from the extracellular to the cytoplasmic space, thereby causing intracellular acidification with resulting cell dysfunction (Rotstein 1993).

A further potential mechanism by which anaerobes may contribute to the pathogenicity of mixed infections, is by virtue of their ability to induce local fibrin deposition (Rotstein & Kao 1988, McRitchie *et al* 1991). Fibrin depositions are capable of capturing bacteria that are not cleared by the lymphatic system or killed by macrophages and leukocytes.

The majority of research into the virulence factors of *Bacteroides* has concentrated on capsular polysaccharides and lipopolysaccharide structures which are discussed in greater detail overleaf.

1.5.5 Capsular Polysaccharides

The purified capsular polysaccharide (CP) of *B. fragilis* has been found to produce abscesses even in the absence of viable organisms (Onderdonk *et al* 1977). Immunisation with a crude polysaccharide has been found to protect against abscess formation (Onderdonk *et al* 1982).

Due to work by Patrick and colleagues we now recognise the fact that a culture of *B. fragilis* has been shown to be morphologically heterogeneous with respect to size and antigenicity of its capsule.

The substitution of Proteose Peptone Yeast extract (PPY) medium for the minimal medium of Van Tassell & Wilkins' (VT) (1978) resulted in greatly enlarged capsules as observed by light microscopy. Accurate quantification of bacteria bearing large capsules was made possible by Percoll density gradient centrifugation (Patrick & Reid 1983).

Light microscopy revealed that on the Percoll gradient, the 0-20% interface enriched for large capsule, the 20-40% for small capsule, 40-60% for a mixture of small capsule and non-capsulate bacteria and the 60-80% interface for non-capsulate bacteria only. Electron microscopy further revealed that the non-capsulate bacteria had an electron dense layer (EDL) outwith the outer membrane. Subsequent studies with monoclonal antibodies (Lutton *et al* 1991, Reid *et al* 1987) revealed that the small capsule was not simply a smaller amount of the large capsule but carried antigenically distinct epitopes.

Recently Tzianabos *et al* (1992) showed that the CP of *B. fragilis* comprises two ionically linked polysaccharides termed polysaccharide A and polysaccharide B which

are present in the ratio 1:3.3. More recently they found that the ability of polysaccharide A and B to induce abscess formation was diminished by alteration of the positive and negative charge (Tzianabos *et al* 1994).

The role of the capsule may be to impair phagocytic uptake and killing by human PMNs probably by mopping up host opsonins. It is known that large capsulate bacteria impairs phagocytic killing but the EDL population is readily phagocytosed *in vitro* although it is resistant to killing by normal human serum (Patrick & Lutton 1990, Patrick 1993). It is also known that *B. fragilis* secretes large amounts of extracellular polysaccharide (EPS) or extracellular vesicles (ECV). ECV can cause haemagglutination and both EPS and ECV can cause the depletion of opsonins and perhaps the activation of complement at a distance from the bacteria (Patrick 1993).

The capsule is not thought to enhance resistance to complement (Reid & Patrick 1984). Previous studies have shown that *Bacteroides* strains isolated from infections are more resistant to complement than those isolated from faeces (Casciato *et al* 1979). Clinical isolates of *B. fragilis* are more resistant to complement than clinical isolates of other *Bacteroides* species (Rotimi & Eke 1984). Recent studies in our laboratory found that *Bacteroides* species became more resistant to complement after growth in VT medium and heat inactivated sheep serum (HISS) as compared to growth in PPY (Allan & Poxton 1994). These differences were not found to correlate with any significant changes in the CP or lipopolysaccharides.

The CP may also evoke production of proinflammatory cytokines (Cross 1994) and directly influence phagocyte movement.

1.5.6 Lipopolysaccharides

Lipopolysaccharides (LPS) or endotoxin is a complex amphipathic macromolecule firmly bound to the outer membrane of Gram-negative bacteria (Fig 1), which is a lipid bilayer containing proteins and phospholipids with large amounts of LPS being found on the outer leaflet. LPS has an important functional role in Gram-negative bacteria. It provides the cell with selective permeability, protects against phagocytosis and is a major bacterial surface antigen (Rycroft 1984, Galanos & Luderitz 1984). It is when LPS is introduced into the bloodstream and interacts with cells of the immune system that it can exert its most powerful biological effects (Table 1) which can lead to irreversible vascular damage, shock and death. The interaction of LPS and the host is discussed in greater detail in section 1.9. What follows is an overview of the structure of LPS and the characteristics of *Bacteroides* LPS.

1.6 GENERAL STRUCTURE OF LPS

LPS comprises three major regions, the O-specific polysaccharide, the core polysaccharide and lipid A (Fig 2). Each of these components is under separate genetic control and structures vary with respect to their biological activity (Luderitz *et al* 1982).

1.6.1 Lipid A

Lipid A is the most conserved region of LPS. It consists of a phosphorylated glucosamine-disaccharide backbone to which long chain fatty acids are bound (Galanos & Luderitz 1984, Doran 1992). The lipid A is embedded in the outer membrane of the Gram-negative cell envelope and is well recognised as being the most toxic component of the LPS molecule. Small changes in the structure of lipid A can lead to a marked reduction in toxicity (Proctor *et al* 1986, Raetz 1993, Astiz *et al* 1994).

Table 1: The biological effects of LPS

Pyrogenicity
Hypotension
Complement activation
Macrophage stimulation
Induction of cytokines
Induction of interferons
Induction of colony stimulating factor
Induction of prostaglandins
Induction of platelet activation factor
Increase adherence to endothelium
PMN stimulation
Priming for superoxide generation and release
Induction of cytokines
B-lymphocytes
B-cell mitogenesis
Release of colony stimulating factors
Epithelial cells
Release of PMN chemotactic factors
Platelets
Induction of platelet aggregation
Serotonin release
Protein kinase C activation

Fig 1: The Gram-negative cell envelope (Taken from Hancock & Poxton 1988)
LP: lipoprotein, LPS: lipopolysaccharide, P: protein, PL: phospholipid.

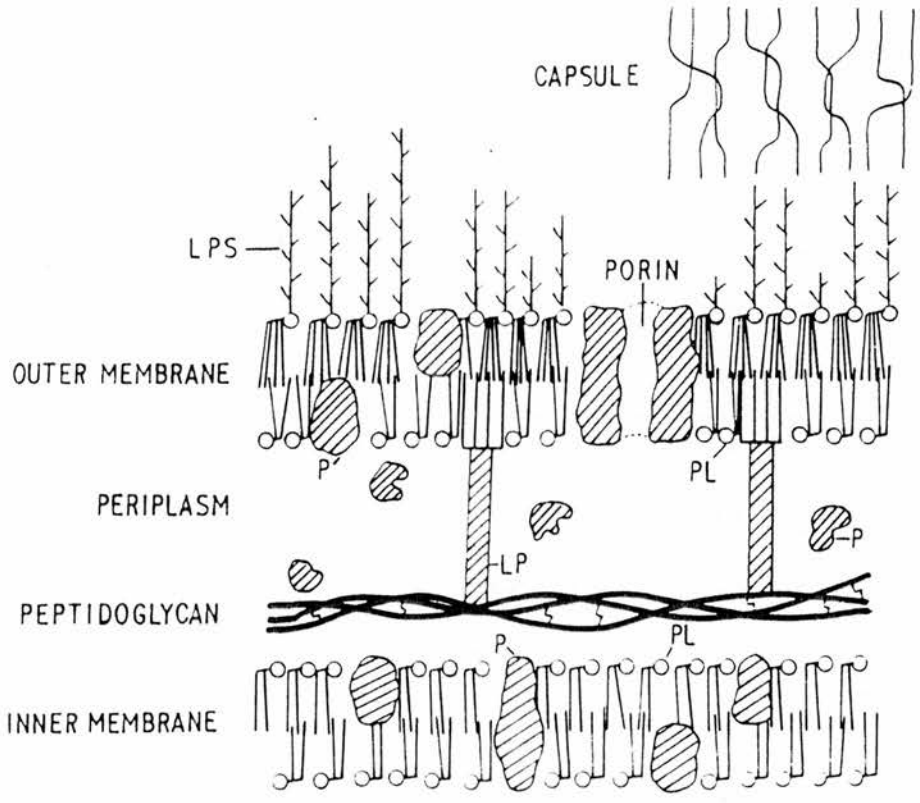
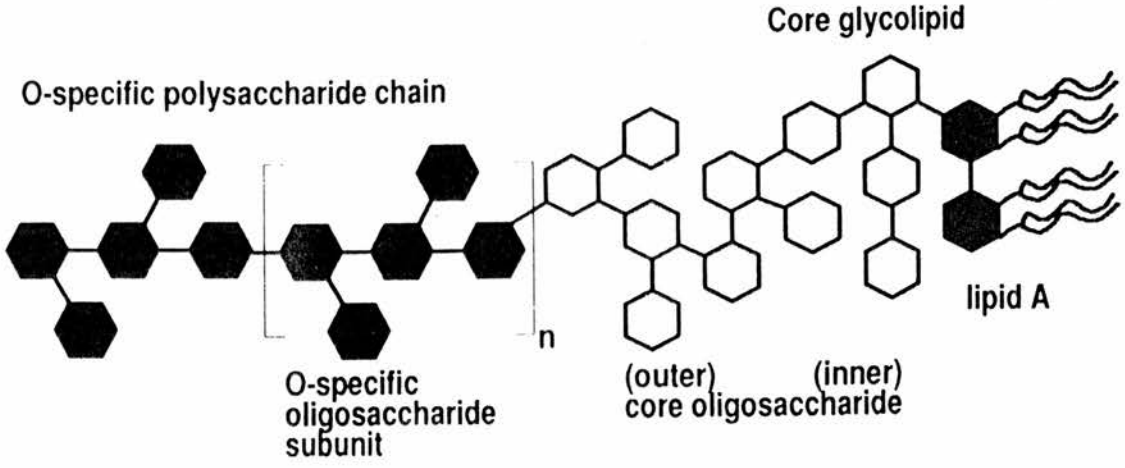


Fig 2: The general structure of LPS



1.6.2 Core Polysaccharides

The core region is divided into inner and outer sub-domains. The outer core is comprised primarily of D-glucose, D-galactose and N-acetyl-D-glucosamine. The inner core contains 3-deoxy-D-manno-octulosonic acid (KDO), heptose as well as phosphate and phosphate bound ethanolamine (Galanos & Ludertiz 1984, Jansonn *et al* 1981). The KDO residue is bound to lipid A by an acid labile ketosidic linkage.

1.6.3 O-Polysaccharides

The O-polysaccharide or " O-antigen" is a highly variable part of the LPS molecule which may or may not be present. It consists of repeating units of oligosaccharide, each comprised of 2-6 monosaccharides. The type and number of repeating units of the O side chain determines the serologic (antigenic) specificity of the O-antigen. The number of repeating units is not fixed, but can show considerable variation under different physiological conditions (McGroarty & Rivera 1990). LPS is often referred to as being "smooth" if the O-antigen is present and "rough" if the O-antigen is absent. Mutant bacteria which lack the O-antigen and produce partial core structures have been discovered (Ra-Re mutants) and have been of great value in biological investigations. It appears that the minimum structural requirements for LPS to mediate endotoxic activity is lipid A linked to KDO.

1.6.4 Bacterial Release of LPS

LPS may be released by viable bacteria during the log phase of growth or in conditions of sub-optimal growth (Tesh & Morrison 1988). LPS is also released after bacterial death as a result of antibiotic therapy or after lysis by the complement cascade. It might be expected that antibiotic treatment may lead to increased levels of LPS during bacteraemia and therefore worsen the clinical situation. However, several studies indicate that this may not be the case *in vivo*, as LPS levels have been found

to decline in the blood following antibiotic therapy (Brandtzaeg *et al* 1989) and any transient increase in LPS levels found have not been associated with poor clinical outcome (Danner *et al* 1991, Shenep *et al* 1988).

1.6.5 Physical State of LPS

LPS is an amphipathic molecule due to its hydrophilic polysaccharide and hydrophobic lipid A. Purified LPS has poor solubility in aqueous solutions because of self aggregation and exists in a highly aggregated form (Galanos & Luderitz 1984). The physical state of LPS is dependent on the presence of inorganic cations and low molecular weight basic amines which neutralise negatively charged groups on the LPS. Electrodialysis of LPS improves its solubility by reducing the amount of positively charged groups. Subsequent conversion of the native acidic LPS to a salt results in a preparation with a lower degree of aggregation (Galanos & Luderitz 1975).

Circulating endotoxin probably never occurs as free LPS, but remains strongly associated with outer membrane proteins. With exposure to serum, the LPS becomes disaggregated and interacts with high density lipoproteins (HDL) (Doran 1992, Ulevitch *et al* 1981). LPS bound to HDL has been demonstrated to be less endotoxic (Cavaillon *et al* 1990). The presentation of LPS in inflammatory serum may be altered due to an increase of circulating lipopolysaccharide binding protein (LBP) which forms high affinity complexes with LPS and presents it to monocytes and macrophages. The interaction between LBP/LPS and monocytes/macrophages is discussed in greater detail in sections 1.9.3 and 1.9.4.

1.7 THE CHARACTERISTICS OF *BACTEROIDES* LPS

Studies on the LPS from *Bacteroides* species have concentrated on *B. fragilis* since it is the most frequently isolated anaerobe in clinical infections. Early studies yielded many confusing and contradictory results which was due in part to the ill-defined LPS preparations used. Studies on the biological activity of *Bacteroides* LPS (Lindberg *et al* 1990, Kasper 1976, Jotwani *et al* 1994) have reached the generally accepted conclusion that it is less endotoxic (100 to 1000-fold less depending on the assay) than enterobacterial LPS.

1.7.1 Nature of the Polysaccharides

For many years it was thought that the LPS from *B. fragilis* was of the rough type with no O-antigen present (Kasper *et al* 1983). However, the presence of repeating oligosaccharide units was reported over the years by other investigators (Cousland & Poxton 1983, Poxton & Brown 1986, Lutton *et al* 1991).

In the paper by Poxton & Brown (1986), the authors concluded that the majority of LPS from *B. fragilis* was of the smooth type with chain lengths of varying molecular mass. The rough form of LPS was common to all strains examined and also contained a low molecular weight common polysaccharide which migrated behind the rough front on polyacrylamide gel analysis which they termed the "common antigen".

Other workers have not been able to detect smooth type LPS (e.g. Weintraub *et al* 1985), and this is due in part to the extraction method employed and to the emergence of the fact that sub-populations which are antigenically distinct exist within a culture of *B. fragilis*.

Lutton *et al* (1991) showed that ladders of repeating polysaccharide subunits were evident mainly in the EDL population. The authors suggest that a variety of populations exist ranging from those where most of the polysaccharide has been released as cell free material, to those in which it remains associated with the bacterial cell.

Sugar analysis of the LPS of *B. fragilis* shows that it contains glucose, galactose, fucose, rhamnose, xylose, glucosamine, galactosamine, ribose, arabinose and mannose (Lindberg *et al* 1990).

There have also been conflicting reports on the presence/absence of L-glycero-D-manno-heptose and KDO which are both present in enterobacterial LPS. Studies by Weintraub *et al* in 1985 failed to detect heptose residues in 17 *B. fragilis* strains tested. KDO is present but in a phosphorylated form, rendering it undetectable in the standard thiobarbituric acid assay (Beckmann *et al* 1989, Kumada *et al* 1993).

1.7.2 Nature of the Lipid A

Variation in the fatty acid content of lipid A between strains of *B. fragilis* was examined by Lindberg *et al* (1990). Only trace amounts of 3-hydroxytetradecanoic acid, the predominating fatty acid in enterobacterial LPS was found. Other major differences between the lipid A of *B. fragilis* and that of *E. coli* were:

(a) *B. fragilis* has an average of 5 fatty acids per lipid A whereas *E. coli* has 6.

(b) *B. fragilis* has long chain fatty acids C15-C17 whereas *E. coli* has C12 -C16.

(c) *B. fragilis* has branching hydroxylated fatty acids and non-hydroxylated fatty acids which are lacking in *E. coli.*

(d) The bis-glucosamine backbone of *B. fragilis* has only one phosphate group, at the reducing end, compared to two in *E. coli.*

It is not known whether the above features are unique to *B. fragilis* LPS or are also present in other *Bacteroides* species.

1.8 THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME

1.8.1 Clinical Features

LPS has been implicated as the principal trigger of the Systemic Inflammatory Response Syndrome (SIRS). This is a characteristic clinical response manifested by two or more of the following symptoms (Darville *et al* 1993, Bone 1993): temperature > 38°C, hypotension, increase in heart rate, increase in respiratory rate, white blood count > 12,000 cells/mm^3 or < 4,000 cells/mm^3.

The term SIRS was proposed by Members of the American College of Chest Physicians in 1992 in an attempt to standardise the definition and terms used to describe this condition. When SIRS is the result of a confirmed infection, it is termed sepsis. Shock associated with SIRS is accompanied with hypotension, declining cardiac output, disseminated intravascular coagulation (DIC) and multi-organ failure (MOF) which can lead onto death.

1.8.2 Incidence of SIRS

Sepsis is a leading cause of death and accounts for estimates of 300,000 cases in the US alone. Mortality from sepsis ranges from 20-50% but in those patients who develop the complications of shock and MOF the mortality rate can reach 90% (Bone 1991).

The increased use of invasive procedures and immunosuppressive treatments are among factors contributing to a growing number of chronically ill and

immunocompromised patients who are at an increased risk from sepsis. Despite the use of more potent and effective antibiotics and technological advances, the mortality rate from septic shock has not changed significantly over the past decade (Bone 1993).

1.8.3 The Source of LPS in Triggering SIRS

Bacteraemia

In a significant proportion of patients with SIRS the source of LPS is thought to arise from an underlying bacteraemia caused by Gram-negative bacteria. *E. coli* is the most common organism isolated with *Klebsiella*, *Enterobacter* and *Pseudomonas* species being encountered less frequently. The incidence of bacteraemia is reported to be as low as 12% (Williats *et al* 1994) to 50% (Bone 1993, Gibb 1993) of cases of SIRS. In a local study by the Sepsis Intervention Group, Edinburgh this figure is around 25% (IR Poxton, Personal Communication). Not all bacteraemias are due to Gram-negative bacteria. In one study of patients with septic shock, 39% had bacteraemias with only 19% caused by Gram-negative organisms (Danner *et al* 1991).

The Gut

In those patients who have SIRS without any bacteraemia, the source of the LPS is thought to arise from the gut. The commensal flora of the gut can be thought as a large pool of endotoxin which may pose a threat to the host. Endotoxin does not appear to absorbed in health (Van Deventer *et al* 1988). However, due to a large fluid loss (e.g. burns, trauma, surgery) there could be a lack of oxygenated blood flow to the gut allowing the mucosal barrier to breakdown. Endotoxin and bacteria could then translocate into the bloodstream via the portal vein and the mesenteric lymph nodes (Runcie & Ramsay 1990).

The translocation of bacteria from the gut to the mesenteric lymph nodes of mono-associated ex-germ free mice was studied by Berg (1995). Studies found that *Ps. aeruginosa*, *Klebsiella* species and *E. coli* were most effective at translocating, with *B. fragilis* and *B. vulgatus* the least effective. There appeared to be a relationship between translocation and sensitivity to killing by O_2. Other viable translocated anaerobic bacteria have not been detected in experimental models to date by other workers. It may not be possible to culture viable anaerobes in such experiments due to killing by O_2, but as other bacteria and latex beads can be seen to translocate it can be assumed the *Bacteroides* or their cellular components could also translocate (Mora *et al* 1991, Mainous *et al* 1991, Wells *et al* 1988). At present no antibody probes have been developed to detect translocated anaerobes or their cellular components.

1.8.4 Which LPSs Are Involved In SIRS?

Despite the fact that LPS is thought to play the central role in the development of SIRS, few studies if any have tried to examine what type of LPS is circulating during SIRS. This is due to the major practical difficulties in actually performing such an assay (Cohen 1989).

If a patient with SIRS has a Gram-negative bacteraemia then LPS from cultured organisms must be considered. But given that the majority of patients with SIRS have no detectable bacteraemia (Bone 1993, Gibb 1993) then LPS derived from organisms in the gut must be considered.

Bacteroides species out number the enterobacteria such as *E. coli* in the gut by approximately 1000-fold (Duerden & Drasar 1991). Although *Bacteroides* LPS is less endotoxic (100 to 1000-fold less depending on the assay) (Lindberg *et al* 1990,

Jotwani *et al* 1994), by simple arithmetic there must be at least as much biological potential from the LPS of *Bacteroides* as from *E. coli*.

1.9 THE PATHOPHYSIOLOGY OF SIRS: HOW LPS INTERACTS WITH THE HOST

1.9.1 General Overview

It is generally agreed that most of the adverse effects of LPS result from its ability to cause the release of various endogenous mediators which act on a number of important biochemical pathways. LPS is a potent activator of B cells and other cells responsive to LPS, including monocytes/macrophages, PMNs, endothelial cells and epithelial cells (Table 1). An overview of the biological responses to LPS in the pathogenesis of SIRS is illustrated in Fig 3. The interaction of LPS with complement and with monocytes/macrophages to produce the cytokine cascade is discussed in more detail below.

1.9.2 Interaction With Complement

LPS can activate both the classical and alternative pathways of complement in the absence of specific antibody. LPS activates the classical pathway by its lipid A region (Vukajlovich 1986) and the alternative pathway by its polysaccharide region (Grossman & Leive 1984). An overview of the complement cascade is illustrated in Fig 4. Activation of the complement cascade results in the lysis of pathogens, the opsonisation of pathogens and the recruitment of phagocytic cells to the site of complement activation (Janeway & Travers 1994).

Fig 3: Biological responses to LPS in the pathogenesis of SIRS (Adapted from Bone 1993, Runcie & Ramsay 1991, Rietschel & Brade 1992).

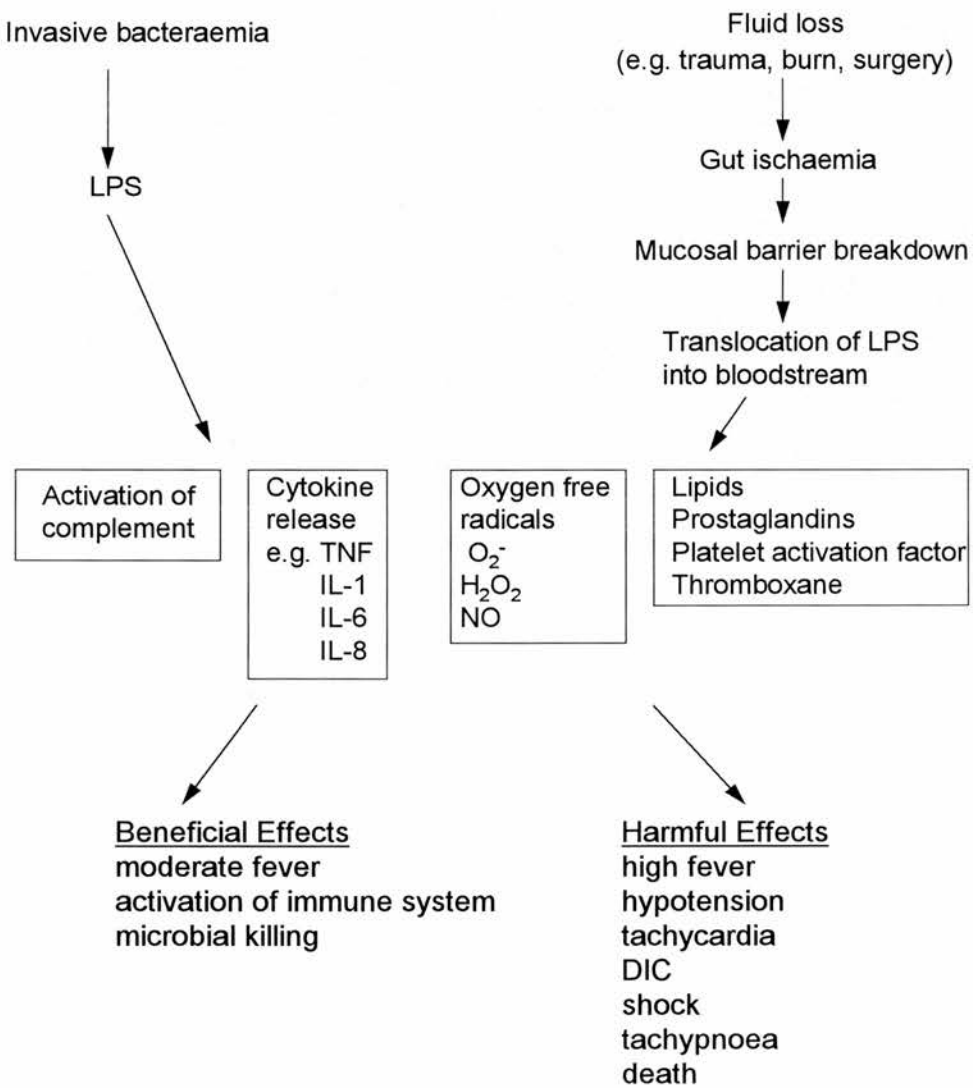
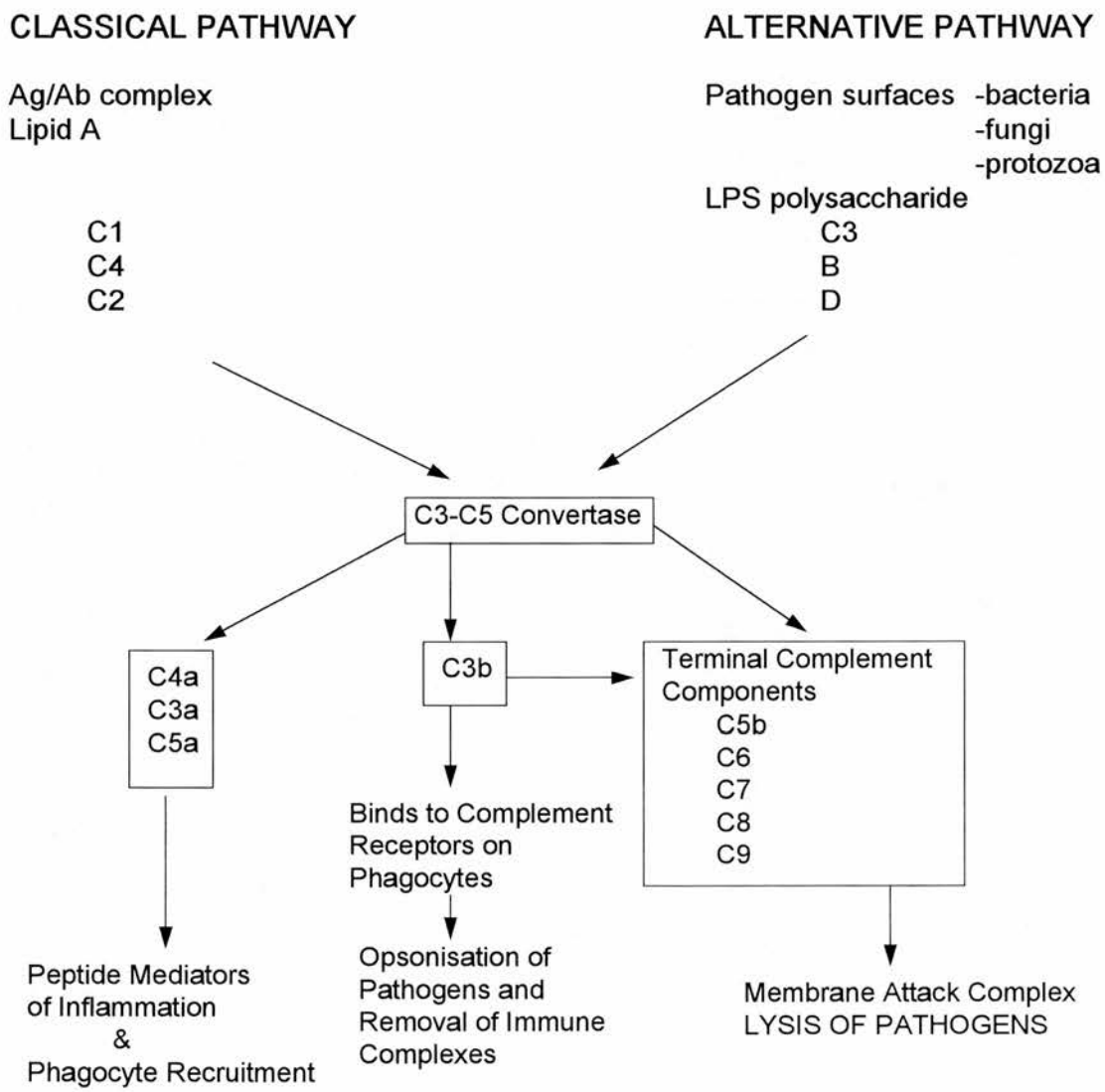


Fig 4: The complement pathway (Adapted from Janeway & Travers 1994).



1.9.3 Interaction With Serum Proteins

It was recognised around two decades ago that serum proteins (such as antibodies, HDL, LBP and sCD14) bind to LPS influencing the subsequent activation of cells responsive to LPS (Ulevitch *et al* 1981, Cavaillon & Haeffner-Cavaillon 1985).

Antibodies

Antibodies to the O Ag, core and lipid A region of *Bacteroides* and *E. coli* LPS are found naturally in the circulation of healthy subjects. Barclay and colleagues have carried out extensive studies investigating the magnitude and kinetics of serum antibody response to LPS core from enterobacteria and closely related species in sepsis patients (Barclay 1990, Windsor *et al* 1993, Barclay *et al* 1989). It was found that non-survivors were more likely to have low endogenous levels of IgG which failed to recover. In both survivors and non-survivors, a sudden consumption of antibody appeared to be indicative of a recent episode of endotoxaemia. In a recent study in our laboratory anti-*Bacteroides* LPS IgG levels were measured in healthy adults and sepsis patients (Allan *et al* 1995). All subjects demonstrated to antibodies to *Bacteroides* LPS and trends in antibody kinetics indicate that *Bacteroides* LPS may be significant in sepsis.

LBP/BPI

Lipopolysaccharide-binding protein (LBP) was discovered during studies into the regulation of LPS binding to HDL (Tobias *et al* 1986). LBP is a 60 kD protein synthesised in hepatocytes as an acute phase reactant. It binds with high affinity to the lipid A region of the LPS (Tobias *et al* 1989). The concentration of LBP is estimated to be 5-10 μg/ml in resting serum and up to 200 μg/ml in acute phase serum from humans (Theofan *et al* 1994).

The ability of LPS to bind to LBP enables it to act as an opsonin (Wright *et al* 1989, Wright 1991). LBP binds to free LPS, Gram-negative bacteria or LPS coated erythrocytes for example and strongly enhances their attachment to macrophages.

Human LBP has been cloned and sequenced and is found to be approximately 44% homologous to human bactericidal/permeability increasing protein (BPI) (Schumann *et al* 1990, Schumann *et al* 1994). BPI is produced in the primary granules of PMNs and like LBP, BPI binds to isolated LPS or to Gram-negative bacteria via lipid A. Some differences between BPI and LBP are summarised in Table 2 (Elsbach & Weiss 1993, Marra *et al* 1992). The main differences are BPI neutralises the ability of isolated LPS to stimulate cells, whereas LBP enhances the effects of LPS on cells. BPI is directly bactericidal whereas LBP functions as an opsonin when it binds to intact bacteria.

Septins

A further group of plasma proteins termed septins that bind to LPS were reported by Wright *et al* (1992). It was proposed that deposition of "septin-1" and "septin-2" on the surface of LPS coated particles activates a latent protease activity required for opsonisation. Because this plasma function could be inhibited by serine protease inhibitors, septins may play a role in presenting free LPS to macrophages.

High Density Lipoprotein

As noted earlier in section 1.6.5 HDL also binds LPS. In a recent study (Wurtel *et al* 1994) it was shown that HDL-like particles reconstituted from purified material cannot neutralise LPS themselves, but the LPS-neutralising capacity of these particles can be realised by the addition of purified LBP. These results suggest that LBP

Table 2: Comparison of the properties of LBP/BPI (Taken from Elsbach & Weiss 1993).

PROPERTY	LBP	BPI
Binding to lipid A/LPS	yes	yes
Binding to Gram-negative bacteria	yes	yes
Synthesis	hepatocyte	PMN precursor
Localisation	plasma	PMN granules
Opsonisation for LPS particles	yes	no
Bactericidal	no	yes
Enhances LPS effects	yes	no
Inhibits LPS effects	no	yes
Molecular weight	60 kD	50 kD
Amino acid sequence homology		> 40%

functions catalytically as a transfer protein that facilitates binding of LPS not only to CD14 (discussed below in section 1.9.4) but also to lipoproteins.

Soluble CD14

LPS can also bind to sCD14 which is discussed in greater detail in section 1.9.4.

When we consider that LPS exists in tight association with bacterial outer membrane proteins and these proteins themselves may be macrophage stimuli, it remains to be determined what role these proteins may have in modulating LPS interactions with serum proteins.

1.9.4 Interaction With CD14 as a Receptor For LPS/LBP Complexes

CD14 is a 55 kD glycosylphosphatidyl inositol (GPI) anchored membrane glycoprotein expressed on monocytes, macrophages and to a lesser extent on PMNs. It is also present as a soluble serum glycoprotein lacking the GPI anchor (Bazil *et al* 1986, Haziot *et al* 1988).

It was determined by Wright *et al* in 1990 that CD14 acts as a receptor for LPS/LBP complexes. CD14 will bind LPS in the absence of LBP but at a much lower rate. The binding of LPS to LBP is thought to occur when LPS concentrations are low (< 1 ng/ml). The interaction between LPS and LBP is not thought to be so crucial when LPS concentrations are high as LPS will be able to interact more easily with CD14 (Hailman *et al* 1994). As mentioned earlier LBP acts catalytically to facilitate binding of LPS to CD14. Recently it has been discovered that the region of CD14 spanning amino acids 57-64 is responsible for binding LPS (McGinley *et al* 1995, Juan *et al* 1995).

CD14 has been shown to play an important role in mediating LPS-induced effects in human monocytes/macrophages, since an anti-CD14 monoclonal antibody (mAb) blocks LPS induced production of TNF-α, IL-6 and IL-8 and oxygen radicals (Chaby & Girard 1993, Ulevitch & Tobias 1994, Manthey & Vogel 1994, Courtier *et al* 1992, Landmann *et al* 1995a). The importance of CD14 in controlling LPS responses was emphasised by Lee *et al* (1992) who transfected the murine 7OZ/3 pre- B cell line with human CD14 cDNA and observed that the amount of LPS required to activate the cells was lowered by up to 10,000-fold.

CD14 may not be the true signalling receptor for LPS as it lacks transmembrane and cytoplasmic regions. Evidence shows that binding of LPS to CD14 is followed by subsequent interaction of additional membrane components that enable transmembrane signalling (Lee *et al* 1993). Whether or not CD14 is internalised together with LPS/LBP remains to be clarified. Studies by Kitchens *et al* (1992) show that uptake of LPS that is dependent on membrane CD14, is independent of the function of this molecule in mediating cell activation. LPS can interact with membrane bound CD14 on monocytes which through the secretion of soluble mediators can activate endothelial and epithelial cells (Pugin *et al* 1993a). The soluble mediators are not yet known and evidence suggests that such an indirect method is more efficient.

Further evidence that CD14 may transmit its signal to an unidentified signalling receptor is due to the fact that cell types that do not express membrane CD14 are still responsive to LPS. Endothelial and epithelial cells appear to be directly stimulated by LPS/LBP complexes binding to soluble CD14 (sCD14) (Pugin *et al* 1993b, Frey *et al* 1992). sCD14 is thought to originate from enzymatic cleavage of membrane bound CD14 via the action of specific proteases (Bazil *et al* 1989). Concentration of sCD14 in normal human plasma have been estimated at 6 μg/ml and LPS and TNF have been

shown to produce dose-dependent increases in sCD14 (Schutt *et al* 1992) An increased amount of circulating sCD14 has been associated with a high mortality in Gram-negative septic shock (Landmann *et al* 1995b).

PMNs possess less CD14 which can be upregulated by IFN-γ, formyl peptides and LPS (Haziot *et al* 1993, Weingarten *et al* 1993). They express and shed CD14, and respond to LPS/LBP complexes via CD14 just like monocytes and macrophages.

1.9.5 Other LPS Receptors

In addition to CD14, other LPS receptors include a 73 kD LPS binding protein, scavenger receptors and the leukocyte antigen CD18.

A 73 kD protein described by Lei & Morrison (1988) present on the surface of murine and human lymphoid and myeloid cells was discovered using a radio-iodinated photoactivatable LPS probe. Further studies using a hamster mAb to this 73 kD protein activates macrophages in a manner that mimics certain actions of LPS, suggesting that this protein may be a functional LPS receptor (Chen *et al* 1990).

The scavenger receptor that binds LPS is found in large amounts in the liver (Kupffer cells and sinusoidal endothelial cells) and is thought to remove endotoxin entering the circulation through pinocytosis and digestion (Wright 1991). It is a trimeric transmembrane glycoprotein composed of cysteine rich carboxy terminal exoplasmic domains connected to a transmembrane domain by a collagen like stalk (Kodama *et al* 1990).

Another well characterised LPS receptor is the CD18 antigen. *E. coli* is bound weakly by macrophages between LPS and adhesion-promoting receptors that share CD18 as

a common β-subunit (Wright & Jong 1986). This then promotes clearance of LPS by phagocytosis and digestion. The portion of LPS recognised by CD18 resides in the lipid A region and *in vitro* studies have shown that anti-CD18 mAb causes heightened TNF secretion in response to low doses of LPS (Wright *et al* 1990).

1.9.6 Production of Cytokines by LPS Stimulated Macrophages and Monocytes

Based on an increasing body of evidence, it is now agreed that many of the effects of LPS are due to the actions of cytokines. Cytokines are proteins or glycoproteins of less than 80 kD that influence the behaviour of the cell producing the cytokine or cell types of a different lineage. They are involved in the development and maintenance of immunity and inflammatory processes. They regulate the amplitude and duration of the response, have many overlapping qualities and function mainly as short range molecules.

Those molecules classed as cytokines, their main source and actions are illustrated in Table 3. Several of the LPS induced cytokines which are thought to play an important role in SIRS and that have been examined experimentally in this thesis are now discussed in more detail.

Tumour Necrosis Factor

Tumour necrosis factor (TNF-α) was initially recognised as its name suggests because of its cytotoxic and anti-tumour effects (Carswell *et al* 1975). It was later found to be identical to a factor called cachectin (Tracey *et al* 1986). TNF-α is produced primarily by monocytes and macrophages but can also be produced by natural killer (NK) cells, PMNs, T lymphocytes, smooth muscle cells and tumour cells. TNF-α is genetically and structurally closely related to TNF-β (lymphotoxin) which is produced by TH_1 lymphocytes. They are both trimers of around 17 kD and TNF-β shares 30%

Table 3: Cytokines - Their main source and actions (Adapted from Janeway & Travers 1994)

Cytokine	Main Producers	Main Actions
IL-1	macrophages, epithelial cells	fever, T cell activation, macrophage activation
IL-2	T cells	T cell proliferation
IL-3	T cells, thymic epithelial cells	synergistic action in haematopoiesis
IL-4	T cells, mast cells	B cell activation, IgG switch
IL-5	T cells, mast cells	eosinophil growth, differentiation
IL-6	T cells, macrophages, neutrophils	acute phase reaction, T/B cell growth and differentiation
IL-7	bone marrow stroma	growth of pre-B and pre-T cells
IL-8	macrophages, monocytes, others	chemotaxis for PMN and T cells
IL-9	T cells	mast cell enhancing activity
IL-10	T cells, macrophages	potent suppresser of macrophage function
IL-11	stromal fibroblasts	synergistic action with IL-3/IL-4 in haematopoiesis
IL-12	B cells, macrophages	activates natural killer cells, induces CD4 T cell differentiation to TH_1 like cells
TNF-α	macrophages, monocytes, PMN, NK cells	local inflammation, endothelial activation
TNF-β	T cells, B cells	kills T cells and fibroblasts, endothelial activation
GMCSF	macrophages, T cells	stimulates growth of differentiation of myelomonocytic lineage
MCP-1	macrophages, others	chemotactic for monocytes
IFN-γ	T cells, NK cells	macrophage activation, increased MHC expression
IFN-α	leukocytes, macrophages	anti-viral, increased MHC class I expression
IFN-β	fibroblasts, macrophages	anti-viral, increased MHC class I expression

Where IL = interleukin, TNF = tumour necrosis factor, GMSCF = granulocyte macrophage colony stimulating factor, MCP-1 = monocyte chemotactic peptide-1 and IFN = interferons.

homology to TNF-α and binds to the same receptors (Darville *et al* 1993, Bendtzen 1988, Rees 1991).

TNF-α plays an important role in the events of SIRS as many of the sequelae of LPS challenge can be reproduced by infusing purified TNF-α into animals and death occurs after administration of high doses of TNF (Tracey *et al* 1986). Passive immunisation of mice with anti-TNF-α antibodies can protect against subsequent challenge with LPS (Beutler *et al* 1985). Increased levels of TNF-α have been found in patients with SIRS and intra-abdominal sepsis (Hamilton *et al* 1992, Michie *et al* 1988, MacDonald *et al* 1990, Endo *et al* 1992).

TNF-α is produced early in the cytokine cascade after stimulation by LPS. To date it appears to be the first cytokine produced in the serum and its production by macrophages does not require the presence of other cytokines (Manthey & Vogel 1992). TNF-α possesses multi-functional activity summarised in Table 4.

The major local effect of TNF-α is to initiate an inflammatory response. It causes vasodilation leading to increased blood flow, an increase in vascular permeability leading to the local accumulation of fluid, as well as the accumulation of immunoglobulin and complement. TNF-α also induces the expression of adhesion molecules that bind to circulating monocytes and PMNs such as P-selectin which appears a few minutes after TNF-α, E-selectin which appears later after a few hours and ICAM-1. These molecules greatly enhance the rate at which these phagocytic cells migrate into the tissues (Janeway & Travers 1994).

Table 4: Multi-functional activity of TNF-α (Adapted from Bendtzen 1988)

Activation *in vitro* of
T, B lymphocytes and NK cells
monocytes
PMNs
endothelial cells
osteoclasts
fibroblasts
hepatocytes
Induction *in vivo* of
fever
wasting
slow wave sleep
anorexia
acute phase protein synthesis
hypotension
capillary leakage

Although TNF-α is important in controlling a local infection, once an infection spreads systemically the results can be devastating. The systemic release of TNF-α causes vasodilation and loss of plasma volume due to increased vascular permeability. This can lead to shock, DIC, MOF and consequently death as previously discussed in section 1.8.

TNF mediates its cellular effects by cross linking to cell surface receptors that bind both TNF-α and TNF-β. Two TNF receptors have been identified as being the 55 kD receptor (TNF r55) and the 75 kD receptor (TNF r75) (Tartaglia & Goeddel 1992). *In vitro* experiments have shown that these receptors can be rapidly shed from PMNs (Ding & Porteau 1992) and slowly from monocytes (Leeuwenberg *et al* 1994). Higher levels of soluble TNF receptors (sTNFr) are found in patients with sepsis (Shapiro *et al* 1993). The role of sTNFr is still speculative but it may play a role in scavenging circulating TNF resulting in a decrease of the inflammatory reaction.

Human TNF r55 and TNF r75 share 28% homology and it has been shown that the TNF r55 binds TNF-α and β with a four-fold lower affinity than the TNF r75 receptor (Tartalgia *et al* 1991). Epithelial cells primarily express the TNF r55 receptor whereas myeloid cells express both the TNF r55 and TNF r75 receptors (Brockhaus *et al* 1990). Recent reports have shown that the signal through the TNF r55 is necessary for most biological functions of TNF (Tartaglia *et al* 1991, Thoma *et al* 1990, Pfeffer *et al* 1993). The role of the TNF r75 in TNF signalling is not well understood but it has been proposed by Tartaglia *et al* (1991) that the TNF r75 acts to capture TNF at the cell surface and thereby increases binding to the lower TNF r55 receptor. It is not known whether the different TNF receptors transduce the same signal or different signals. It is also not known whether the multiple functions of TNF are mediated through one or both receptors.

Interleukin-6

Interleukin-6 (IL-6) exists in nature as a monomeric 26 kD protein. On the basis to its various activities it was previously known as β_2-interferon, B-cell stimulatory factor 2, hybridoma/plasmacytoma growth factor and monocyte granulocyte inducer type 2 (Hirano *et al* 1990, Tafuto *et al* 1994). IL-6 is produced by monocytes, macrophages, B/T lymphocytes, endothelial cells, keratinocytes, fibroblasts, bone marrow stroma cells and various tumour cells (Bendtzen 1988, Tafuto *et al* 1994). IL-6 has multi-functional activity summarised in Table 5 but unlike TNF-α, exogenous IL-6 administration does not cause haemodynamic compromise, regardless of the amount given to experimental animals (Preiser *et al* 1991).

One of the most important activities of IL-6 is the initiation of the acute phase response. It involves a shift in the proteins secreted by the liver into the blood and results from the actions of IL-6 on hepatocytes. IL-6 induces the production of acute phase proteins including serum amyloid protein, C-reactive protein (CRP), fibrinogen and mannose-binding protein (MBP). CRP binds phosphorylcholine and MBP binds mannose residues on bacterial surfaces. Both act as opsonins and activate complement to lyse bacteria (Janeway & Travers 1994, Darville *et al* 1993, Tafuto *et al* 1994).

Increased levels of IL-6 have been found in sepsis (Hack *et al* 1989) and studies have shown that anti-IL-6 monoclonal antibodies protects from lethal *E. coli* infection as well as from lethal amounts of TNF (Starnes *et al* 1990). IL-6 acts on its target cells via a specific dimeric 80 kD IL-6 receptor. Binding is followed by association of the 80 kD protein with a 130 kD transmembrane protein providing signal transduction (Boer & Herrmann 1991).

Table 6: Multi-functional activity of IL-6 (Adapted from Bendtzen 1988)

Activation *in vitro* of
T, B lymphocytes and NK cells
monocytes
PMNs
osteoclasts
hepatocytes
nerve cells
Induction *in vivo* of
fever
low wave sleep
anorexia
acute phase proteins

Abnormal production of IL-6 has also been suggested to be involved in the pathogenesis of glomerulonephritis, multiple myeloma, Hodgkin's lymphoma and Rheumatoid arthritis (Tafuto *et al* 1994, Bendtzen 1988).

Interleukin-1

Interleukin-1 (IL-1) is produced by monocytes, macrophages, NK cells, PMNs, B cells, dendritic cells and endothelial cells. IL-1 consists of IL-1 α and IL-1 β which are structurally related polypeptides that show 25% amino acid homology. Both are produced as 31 kD precursors that are subsequently cleaved into 17 kD molecules. IL-1 α remains in the cytosol of cells or is associated with the cell membrane whereas IL-1 β is secreted (Bendtzen 1988). IL-1 has never been shown to be directly lethal to animals as has TNF-α (Tracey *et al* 1987) but produces the same effects as TNF-α and IL-6 (Tables 4, 5) to the same or lesser extent *in vivo* and *in vitro*.

Interleukin-8

Interleukin-8 (IL-8) exists as a dimeric 83 kD protein which is produced by monocytes, macrophages, hepatocytes, PMNs, dermal fibroblasts and endothelial cells in response to LPS, IL-1β and TNF-α (Janeway & Travers 1994, Liebler *et al* 1994, Nielson *et al* 1994). IL-8 can also be produced by epithelial cells in direct response to bacterial entry (Eckmann *et al* 1993). This may serve as an early signalling system to host immune and inflammatory cells in the underlying mucosa following bacterial entry.

Primarily, IL-8 is a chemoattractant for PMNs, inducing them to leave the bloodstream and migrate into the surrounding tissues. Further biological functions of IL-8 *in vivo* include induction of lymphocyte infiltration, increase of vascular

permeability in the presence of prostaglandin E and destruction of the synovial membrane (Neilson *et al* 1994).

IL-8 belongs to a common supergene C-X-C family, in which the first two cysteines are separated by a single amino acid residue. IL-8 shares 21% homology at the amino acid level with monocyte chemotactic peptide-1 (MCP-1) which is a potent chemoattractant for monocytes. MCP-1 belongs to the supergene C-C family in which the first two amino acids are in juxtaposition (Baggioloni *et al* 1994).

It has been found that levels of circulating IL-8 are elevated in patients with sepsis and this level is further elevated in those patients with shock (Hack *et al* 1992, Solomkin *et al* 1994).

1.9.7 Production of Cytokines by LPS Stimulated Polymorphonuclear Neutrophils

Polymorphonuclear neutrophils (PMN) are the most common leukocytes in human blood and are the first cells to migrate into tissues in response to invading pathogens. Since PMNs are short lived cells, terminally differentiated and incapable of proliferation, their ability to synthesise cytokines might be viewed of little significance (Janeway & Travers 1994, Cassatella 1995). However, in the last few years several groups have shown that PMNs can release cytokines including TNF-α, IL-1 β, IL-1 receptor antagonist and IL-8 but on a scale much lower than monocytes and macrophages (reviewed in Cassatella 1995).

As previously discussed in section 1.9.4, PMNs possess less CD14 receptors than monocytes and macrophages, and respond to LPS/LBP complexes to produce TNF-α (Haziot *et al* 1993, Weingarten *et al* 1993, Dubravec *et al* 1990). PMNs can secrete

TNF-α in response to high doses of LPS (5 μg/ml) compared to monocytes and macrophages which can respond to lower levels of LPS (< 1 ng/ml). *In vivo* PMNs might prove to be a significant source of cytokines, that may influence the direction and evolution of SIRS.

1.10 CYTOKINE INDUCTION BY GRAM-POSITIVE BACTERIA AND OTHER BACTERIAL STRUCTURES

Although LPS is the principal trigger of SIRS and the cytokine cascade, there is increasing evidence to suggest that components of Gram-positive cell walls and other bacterial products stimulate the release of cytokines from macrophages.

It has been shown that purified cell walls from Gram-positive bacteria, and peptidoglycan and teichoic acid from *Staphylococcus epidermidis* stimulate the release of IL-6 and TNF-α on a magnitude 100 times less than *E. coli.* (Heumann *et al* 1994, Mattsson *et al* 1993, Lichtman *et al* 1993). It is not known what role serum proteins play in the induction of cytokine release by Gram-positive cell wall components.

Fimbriae from *Porphyromonas gingivalis* have also been shown to induce production of IL-1 β, IL-6, IL-8 and TNF-α in human blood monocyte/macrophage cultures (Ogawa *et al* 1994b). It is also proposed that the CP from *B. fragilis* may induce cytokine release (Cross 1994).

1.11 TREATMENT OF SIRS

As previously discussed, SIRS results from the interactions of LPS with the host. Accordingly, therapies aimed at inhibiting or neutralising endotoxin or the cytokine

cascade have been extensively explored. As yet no anti-LPS or anti-cytokine treatment of SIRS and shock have proven any real benefit. What follows is a discussion of some of the therapies that have been approached.

1.11.1 Anti-Endotoxin Strategies

Table 6 outlines several of the anti-endotoxin approaches to the treatment of SIRS.

Antibodies to endotoxin

Most therapeutic efforts have focused on passive immunotherapy against endotoxin. Several monoclonal antibodies (mAbs) that bind to the core and lipid A region of LPS (the region most responsible for the toxicity of LPS and has a highly conserved structure) have been generated. One of these, HA-1A (Centoxin), a human anti-lipid A IgM mAb caused a great deal of controversy. The efficacy of this antibody was reported in 1991 by Ziegler *et al.* During a controlled trial of HA-1A in the treatment of patients with SIRS, no difference in 28 day mortality was seen in the whole group of 543 patients treated. But in those patients who were subsequently proven to have Gram-negative bacteraemia there was a significant reduction in 28 day mortality from 49% to 30% in those that received HA-1A. These results prompted the release of HA-1A on the market in some European countries. Reports of an excess mortality in treated patients with non-Gram-negative bacteraemia resulted in HA-1A being withdrawn from sale (Baumgartner 1994, Evan & Cohen 1993).

Table 6: Anti-endotoxin therapies for treating SIRS (Taken from Corriveau & Danner 1993).

- Non antibody agents that bind endotoxin
  - Cationic peptides
    - Colistin, colistin nonapeptide
    - Polymyxin B, polymyxin B nonapeptide
    - Polymyxin B-dextran 70
  - Cationic proteins
    - Lipopolysaccharide binding protein
    - Bactericidal/permeability increasing protein
    - Tachyplesin (anti-endotoxic factor from horseshoe crab amebocytes)
  - Lipoproteins
    - High-density lipoprotein
    - Low-density lipoprotein
- Analogues of lipid A
  - Derivatives that induce tolerance
    - Monophosphorylated lipid A
    - Sandoz compound 953 (SDZ MRL 953)
  - Direct endotoxin antagonists
    - Lipid X
    - Lipid IV_A
    - Diphosphoryl lipid A from *Rhodopseudomonas sphaeroides*
- Anti-endotoxin antibodies
  - O specific
  - Core specific
  - Lipid A specific
    - E5 (Xomen)
    - HA-1A (Centoxin)

Non-antibody agents that bind endotoxin

Polymyxin B binds tightly to the lipid A region of LPS and has been shown to neutralise many of its activities including lethality (Corriveau & Danner 1993, Flynn *et al* 1987). Most investigations have concluded that polymyxin B needs to be premixed with endotoxin *ex vivo* or given simultaneously to be protective and has never been evaluated in a clinical trial (Corriveau & Danner 1993).

Theofan and colleagues (1994) cloned and expressed human recombinant LBP (rLBP) as well as a truncated version $rLBP_{25}$. Both bound LPS with the same affinity but whereas rLBP was able to mediate LPS induced TNF secretion by monocytes, $rLBP_{25}$ was inactive. This suggests that the LPS binding site resides in a different region of the protein from the CD14 stimulatory activity and that $rLBP_{25}$ may be of use in the treatment of endotoxaemia.

BPI has been investigated as a potential anti-endotoxin agent. It binds to Gram-negative bacteria and LPS via their lipid A moieties. It has been shown to inhibit the production of TNF in whole human blood and PMN priming. A recombinant amino-terminal fragment of BPI ($rBPI_{23}$) has been shown to retain the properties of the parent protein and is currently being developed for treatment (Gazzano-Santoro *et al* 1992).

Analogues of lipid A

Non-toxic derivatives of lipid A that reduce tolerance in animals include monophosphorylated lipid A (MPL) and Sandoz compound 953 (SDZ MRL 953) (Corriveau & Danner 1993, Lam *et al* 1991). Other structural derivatives of lipid A that have been found to antagonise the actions of LPS *in vitro* and protect endotoxin-challenged animals from death include lipid X (a monosaccharide precursor in the

biosynthetic pathway of lipid A) (Danner *et al* 1987), lipid IV_A (Kovach *et al* 1990) and the diphosphoryl lipid A of *Rhodopseudomonas sphaeroides* (RsDPLA) (Takayama *et al* 1989). Lipid X has been shown to block PMN priming and lipid IV_A and RsDPLA have been shown to block endotoxin induced TNF production.

1.11.2 Blocking The Cytokine Cascade

Since SIRS is characterised by a complex interplay of cytokines it was logical to try blocking cytokine overproduction. Potential agents for blocking the cytokine cascade are illustrated in Table 7.

Table 7: Blocking the cytokine cascade (Taken from Darville *et al* 1993).

Cytokine	Potential Blocking Agent
TNF	anti-TNF antibodies soluble TNF receptors TNF-receptor Tc chimeric proteins
IL-1	IL-1 receptor antagonist
IL-6	anti-IL-6 antibodies

Since IL-1 and TNF-α act synergistically in shock, preventing either cytokine reduces the effect of the other. No adequate clinical trial addressing the efficacy of an anti-TNF mAb in human sepsis has yet been published (Darville *et al* 1993, Mercier 1993).

In addition to antibodies the discovery of natural antagonists of cytokine action has led to further therapeutic possibilities. These include a soluble form of the TNF receptor which may act in scavenging circulating TNF resulting in a decrease of an inflammatory reaction. Recent studies have shown that the human recombinant p55 sTNFr is a better candidate for the development of an anti-inflammatory agent (Hale *et al* 1995). The IL-1 receptor antagonist (IL-1 RA) is a naturally-occurring protein

which binds to the human IL-1 receptor but has no agonist activity. It has been shown to reduce mortality rates in a rabbit model of sepsis even when it was given after the onset of shock (Aiura *et al* 1991). However, the results of a phase III clinical trial has shown there was no decrease in mortality in treated patients (Hannum *et al* 1990).

A lack of cytokine activity may be detrimental. It has not been determined whether early intervention in the cytokine cascade would compromise longer term inflammatory response or tissue repair. Our understanding of SIRS is by no means complete. The high mortality rate associated with severe sepsis and SIRS will undoubtedly lead to broad application of immunotherapies if they prove to be effective.

AIMS OF THIS THESIS

The objective of this thesis was to re-assess the biological activity of *Bacteroides* LPSs and evaluate their possible role in sepsis. The following main areas of research were covered:-

1. To investigate the biological activities of *Bacteroides* LPSs which had been extracted by three different methods compared to an *E. coli* O18K$^-$ LPS control.

2. To try and relate biological activity to sub-populations, Percoll profiles, chemotype and chemical composition of *Bacteroides* LPS and CPs.

3. To examine the capacity of *Bacteroides* LPS and CP to induce various cytokines from three different cell populations, with particular emphasis on the role of CD14 in cell activation. Inhibition of cytokine production by anti-*Bacteroides* mAbs will be attempted.

4. To examine how mitogenic *Bacteroides* LPS and CP are to LPS-responder mice.

Materials and Methods

2.1 REAGENTS

All reagents were obtained from BDH, Poole, England, UK unless otherwise indicated.

2.2 BACTERIAL STRAINS

Table 1 overleaf lists all the Gram-negative organisms used. Strains were maintained as lyophilised cultures. MPRL 1522 could not be distinguished between *B. merdae* or *B. distasonis* due to the insensitivity of the arabinogalactan fermentation test.

2.3 BACTERIAL CULTURE MEDIA

(a) Nutrient broth (Gibco, Paisley, Scotland) (NB) was prepared and filter sterilised by the Blood Transfusion Service, Protein Fractionation Centre, Edinburgh.

(b) Nutrient agar was Columbia agar base (Oxoid, Basingstoke, England).

(c) Proteose Peptone Yeast extract (PPY) medium pH 7.1 contained per litre: Proteose peptone (Oxoid) 20 g, yeast extract (Difco, Detroit, USA) 10 g, NaCl 5 g, cysteine HCl (3.75% w/v solution) 20 ml, sodium carbonate (2% w/v solution) 20 ml, haemin/menadione (250 μg/ml: 50 μg/ml solution) 20 ml and 940 ml of pyrogen free water (PF-H_2O).

(d) Van Tassell and Wilkins' (VT) medium was prepared as described in Van Tassell and Wilkins (1978). It contained per litre: $(NH_4)_2SO_4$ 2 g, sodium citrate 0.5 g, vitamin B12 5 μg, KH_2PO_4 7 g, K_2HPO_4 8 g, $MnCl_2.4H_2O$ 10 mg, $MgCl_2.6H_2O$ 20 mg, $FeCl_3.6H_2O$ 0.3 mg, $CaCl_2.2H_20$ 30 mg, $NaHCO_3$ 4 g, cysteine HCl 0.5 g, glucose 10 g, haemin 5 mg and resazurin 1 mg.

Table 1: Bacterial strains

Species	Strain	Source/origin	Used in sub-population experiments
B. fragilis NCTC 9343	MPRL 1669	Appendix abscess	Yes
B. fragilis GNAB 4	MPRL 1504	Wound	Yes
B. fragilis	MPRL 1652	Faeces	
B. fragilis	MPRL 1978	Blood	
B. vulgatus NCTC 11154	MPRL 1726	?	Yes
B. vulgatus	MPRL 1651	Faeces	Yes
B. vulgatus	MPRL 1985	Blood	Yes
B. thetaiotaomicron NCTC 10582	MPRL 1720	Faeces	Yes
B. thetaiotaomicron	MPRL 1959	Blood	
B. distasonis ATCC 8503	MPRL 1667	?	Yes
B. caccae ATCC 43185	MPRL 2278	Faeces	
B. merdae ATCC 43184	MPRL 2277	Faeces	
B. merdae or distasonis	MPRL 1522	Faeces	
B. caccae	MPRL 1555	Wound	Yes
B. ovatus ATCC 8483	MPRL 1709	?	
B. ovatus	MPRL 2370	Blood	Yes
B. thetaiotaomicron	MPRL 1987	Blood	
B. uniformis ATCC 8492	MPRL 1721	?	Yes
B. uniformis	MPRL 1542	Faeces	
B. variabilis VPI 11368	MPRL 1724	?	Yes
B. variabilis	MPRL 2244	?	
B. stercoris ATCC 43183	MPRL 2276	Faeces	
B. eggerthii NCTC 11155	MPRL 1668	Faeces	Yes
B. eggerthii	MPRL 1523	Faeces	
B. eggerthii	MPRL 1216	Appendix	
E. coli O18K$^-$	MPRL 1275	Dr A. Cross, Walter Reed Army Institute, Washington D.C., U.S.A.	

(e) Van Tassell and Wilkins' medium containing 50% heat-inactivated sheep serum (VT + 50% HISS). Filter-sterilised sheep serum was obtained from the Moredun Research Institute, Edinburgh, and was heat inactivated by heating at 56°C for 60 min. Sheep serum was stored at -20°C until use.

2.4 BACTERIAL CULTURE

The purity of all cultures was determined by wet film, Gram stain and a 48 h purity plate.

2.4.1 Anaerobic Growth

(a) On a small scale *Bacteroides* species were grown in PPY, VT or VT + 50% HISS in an anaerobic cabinet (Don Whitley, Shipley, UK) at 37°C in an atmosphere of 10% H_2, 80% N_2 and 10% CO_2. *E. coli* O18K$^-$ was grown in NB and incubated at 37°C in an atmosphere of 10% H_2, 80% N_2 and 10% CO_2. Cells were either used immediately or harvested by centrifugation at 10,000 g for 15 min before washing twice in phosphate buffered saline (PBS) at 10,000 g for 15 min before use.

(b) On a large scale *Bacteroides* species were grown in PPY or VT, and *E. coli* O18K$^-$ was grown in NB in a 16 l capacity LH fermenter (LH Engineering, Stoke Poges, UK) at 37°C in an atmosphere of 100% N_2. Cells were harvested by centrifugation at 15,000 g using a KSB continuous flow system (Dupont UK Ltd) before washing twice in PBS at 10,000 g for 15 min. The bacterial pellet was lyophilised and stored at -20°C for further use.

2.4.2 Aerobic Growth

(a) On a small scale *E. coli* O18K$^-$ was grown in NB and incubated statically at 37°C in normal atmospheric air. Cells were used immediately or harvested as described above.

(b) On a large scale, *E. coli* O18K$^-$ was grown in NB in a 16 l capacity LH fermenter at 37°C in an atmosphere of 50% pO_2 saturation. Cells were harvested as described above.

2.5 EXTRACTION OF LIPOPOLYSACCHARIDES

2.5.1 Phenol-Water (PW) Method

LPS was extracted by the method of Westphal & Luderitz (1954) as described in Hancock & Poxton (1988). A finely divided lyophilised bacterial mass was resuspended to a concentration of 5% w/v in PF-H_2O and an equal volume of 90% w/v phenol was heated to 67°C in a water bath. Both solutions were mixed and stirred at 67°C for 15 min. The mixture was transferred to glass 50 ml centrifuge tubes and cooled on ice to allow phase separation. The tubes were then centrifuged at 10,000 g for 15 min to complete separation of the phases. The upper aqueous phase containing the LPS was carefully removed and transferred to dialysis tubing (Medical International Ltd, London) (washed and boiled for 10 min in PF-H_2O) and dialysed against running tap water overnight, until the smell of phenol was no longer detectable. The dialysed extract was then concentrated to approximately one-fifth of its original volume by rotary evaporation. This was proceeded by ultra centrifugation (OTD65B, Dupont) of the solution at 100,000 g for 3 h. The resulting pellet was resuspended in PF-H_2O and recentrifuged. The final pellet was suspended in a small amount of PF-H_2O, lyophilised and weighed. The LPS was stored at -20°C until required.

2.5.2 Phenol-Chloroform-Petroleum (PCP) Method

LPS was extracted by the method of Galanos *et al* (1969) as described in Hancock & Poxton (1988). The extraction solvent (PCP) consisted of 90% phenol, chloroform

and petroleum spirit (40°- 60° b.p.) in the ratio 2:5:8 by volume. Lyophilised bacteria were resuspended in PCP at approximately 25% w/v, stirred for 2 min below 20°C and centrifuged at 10,000 g for 15 min. The supernate was filtered through Whatman No 1 filter paper into a round-bottomed flask. The pellet was re-extracted and the filtered supernates pooled. Chloroform and petroleum were removed from the supernate by rotary evaporation. LPS was precipitated by the addition of six volumes of diethyl ether/acetone (1:5 by volume) to one volume of phenol solution. After standing for 1 h the LPS was sedimented by centrifugation at 5,000 g for 10 min. The pellet was washed three times in diethyl ether/acetone and the final pellet dried under vacuum (by water pump) until the smell of ether/acetone was no longer detectable. The pellet was resuspended in 5 ml of PF-H_2O and LPS recovered by ultra centrifugation at 100,000 g for 4 h. The resulting LPS was taken up in a small amount of PF-H_2O, lyophilised, weighed and stored at -20°C until required.

2.5.3 Triton-Mg^{2+} (Triton) Method

LPS was extracted by the method of Uchida & Mizushima (1987). The following method quotes volumes for 1 g dry cell. Volumes were scaled up accordingly.

To lyophilised bacteria, 22 ml of PF-H_2O was added, mixed and followed by successively adding and mixing 4 ml 100 mM Tris-HCl pH 8, 4 ml 0.5 M $MgCl_2$ and 10 ml 8% Triton X-100. Finally, 10 ml of ethanol was added, the tube sealed and boiled at 100°C for 10 min. After cooling, the mixture was centrifuged at 100,000 g for 90 min at room temperature and the precipitate washed once in 40 ml of 10 mM Tris-HCl pH 8 containing 10 mM $MgCl_2$. To the washed precipitate, 10 ml of PF-H_2O, 10 ml 0.2 M EDTA pH 8, 10 ml 2 M NaCl and 10 ml 8% Triton X-100 was added, mixed well and incubated for 37°C for 60 min with agitation. The mixture was centrifuged at 15,000 g for 15 min at room temperature, the supernate collected and

recentrifuged as before. To the supernate, 4 ml 1 M $MgCl_2$ was added while stirring and heated to 37°C for 60 min. The resultant opaque solution was immediately centrifuged at 100,000 g for 90 min at 15°C and the resulting clear precipitate washed once in 40 ml 10 mM Tris-HCl pH 8 containing 10 mM $MgCl_2$. The resultant precipitate was lyophilised, weighed and stored at -20°C until required.

2.5.4 Proteinase K Digestion Of Bacteria For The Preparation Of LPS

Proteinase K digestion of bacteria was used to prepare LPS for analysis by polyacrylamide electrophoresis (PAGE). Bacterial cultures were harvested by centrifugation and washed twice in PBS. The density of washed bacteria was adjusted to an absorbance at 525 nm of between 0.5 and 0.6. Bacterial suspension (1-5 ml) was transferred to an Eppendorf tube and the bacteria sedimented by micro-centrifugation at 10,000 g for 3 min. The pellet was resuspended in 50 µl of single strength PAGE sample buffer (see section 2.9) and heated at 100°C for 10 min. After cooling, 10 µl of sample buffer containing 25 µg of proteinase K (Sigma protease Type X1, St Louis, USA) (2.5 mg/ml in sample buffer) was added followed by incubation in a 60°C water bath for 60 min. Samples were stored at -20°C until required.

2.6 PURIFICATION OF LIPOPOLYSACCHARIDE FROM PROTEIN CONTAMINATION

Native LPSs (5 mg/ml) were made free from any protein contamination by treating with proteinase K (20 µg/ml) by heating at 65°C for 2 h. Proteinase K was removed by two washes with PF-H_2O at 100,00 g.

2.7 DEIONIZATION OF LIPOPOLYSACCHARIDES BY ELECTRODIALYSIS

Deionisation of LPS was based on the method described in Hancock & Poxton (1988). LPS was suspended in PF-H_2O to approximately 5-10 mg/ml and placed in a three-chambered electrodialysis cell (ISCO, Lincoln, Nebraska, USA), and distilled water added to the two electrode chambers. A voltage of up to 500 V was maintained across the cell. The contents of the chambers was replaced several times over 3-4 h when the pH in the cathodic chamber rose. The precipitated free acid form of the LPS (deionised) was recovered from the anodic side of the cell and suspended in PF-H_2O. Deionised LPS was solubilised by neutralisation with NaOH.

2.8 PREPARATION OF CAPSULAR POLYSACCHARIDE FROM *B. FRAGILIS* NCTC 9343

The method of Pantosti *et al* (1991) was followed. *B. fragilis* NCTC 9343 was grown up in 4 l of PPY at 37°C, 5% CO_2. Bacterial cells were suspended in 100 ml of PF-H_2O and an equal volume of 75% w/v phenol was added. The mixture was stirred at 68°C for 30 min and cooled on ice. Phases were separated by centrifugation at 10,000 g for 15 min and the aqueous layer removed. The phenol-water extraction step was repeated and the aqueous layers pooled. An equal volume of ether was added to the aqueous layers to remove the phenol and the upper ether layer removed. The lower layer was concentrated on a rotary evaporator and dialysed against distilled water and lyophilised.

Volumes of the lyophilised material were suspended in 0.1 M sodium acetate buffer (pH 4.5) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$, and treated with 0.5 mg DNase (DN-25, Sigma) and 2.5 mg RNase (Type 1-A, Sigma) at 37°C for 2 h. This treatment was then repeated overnight at 37°C. The pH of the solution was adjusted

to pH 7.0 with NaOH and treated with 5 mg of Pronase (Sigma type 70,000 PUK units per g) at 37°C for 2 h. This treatment was then repeated overnight at 37°C. The material was brought to a concentration of 80% v/v in ethanol and allowed to stand at 4°C overnight. The alcohol insoluble precipitate was recovered by centrifugation and dissolved in a buffer containing 0.5% sodium deoxycholate, 50 mM glycine and 10 mM EDTA, pH 9.8. A volume of this solution (3 ml) was loaded onto a Sephacryl column (S-300) (2.5 cm x 30 cm) (Pharmacia, Uppsala, Sweden) which had been equilibrated in the deoxycholate buffer, pH 9.8. Fractions (1.8 ml) were collected and examined for capsular polysaccharide by PAGE and staining with silver. Fractions that contained capsular polysaccharide and low molecular mass LPS were pooled, precipitated in 80% v/v ethanol and dialysed extensively against distilled H_2O before lyophilising.

The capsular polysaccharide (4.2 mg) was heated at 100°C for 1 h in 10 ml of 5% acetic acid. The acetic acid was removed by rotary evaporation and the precipitate dissolved in 3 ml of 50 mM Tris-HCl buffer, pH 7.3 and loaded onto a column (1.5 cm x 15 cm) of DEAE-Sephacel (Pharmacia) which had been equilibrated in the 50 mM Tris-HCl buffer. Buffer (100 ml) was washed through the column and 5 ml fractions collected. The material bound to the column was eluted with a linear gradient of 0 to 0.5 M NaCl in the 50 mM Tris-HCl buffer collecting 5 ml fractions. Fractions were examined by PAGE stained with silver. Material obtained after elution with the 50 mM Tris-HCl buffer was termed polysaccharide A. Material obtained after elution with low concentrations of NaCl was termed polysaccharide B and material obtained after elution with high concentrations of NaCl was considered a mixture of polysaccharide A and B.

Samples were desalted by running 200 µl samples through disposable polystyrene columns (Pierce & Warner, Luton, England) that had been packed with Sephadex G-25 (Pharmacia) and calibrated with Dextran-blue and NaCl. Samples were pooled and lyophilised.

2.9 PREPARATION OF SAMPLES FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Sample buffer (pH 6.8) contained 0.0625 M Tris in which 2% w/v SDS, 10% v/v glycerol, 1% v/v 2-mercaptoethanol and 0.001% bromophenol blue were present. Double strength sample buffer (pH 6.8) was made as above with double the concentration of all constituents.

Proteinase K digests were prepared as described in section 2.5.4 and samples were loaded onto gels at 10 µl per track for silver staining and Coomassie blue staining.

LPS samples prepared by the PW, PCP and Triton-Mg^{2+} or capsular polysaccharides were suspended in PF-H_2O to 1 mg/ml and an equal volume of double strength sample buffer added. Samples were boiled for 10 min and loaded onto gels at 10 µl per track for silver staining and Coomassie blue staining.

2.10 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE was performed on acrylamide slab gels using the buffer system of Laemmli (1970) as described in Hancock & Poxton (1988). PAGE of LPS samples were run on 14% non-SDS polyacrylamide gels. The following buffers and solution were used:

(a) Separating gel buffer (double strength, pH 8.8) consisted of 0.75 M Tris-HCl.

(b) Stacking gel buffer (double strength, pH 6.8) consisted of 0.25 M Tris-HCl.

(c) Acrylamide stock solution (40% w/v) contained 100 g acrylamide and 2.7 g methylene bis acrylamide made up to 250 ml with distilled water.
(d) Electrode buffer (pH 8.3) consisted of 0.025 M Tris, 0.192 M glycine and 0.1% w/v SDS.

A 14% separating gel consisted of 3.45 ml distilled water, 17.5 ml separating buffer and 12.25 ml acrylamide solution which was deaerated under vacuum prior to addition of 0.05 ml TEMED and 1.75 ml ammonium persulphate (15 mg/ml). The solution was poured between glass plates (160 mm x 125 mm x 1.5 mm) which had been wiped clean with methanol and sealed with molten Vaseline. The gel was overlaid with water-saturated butan-2-ol and allowed to set. After removal of the butan-2-ol, a 4% stacking gel (consisting of 3.5 ml distilled water, 5 ml stacking buffer and 1 ml acrylamide solution prepared as before prior to addition of 0.02 ml TEMED and 0.5 ml ammonium persulphate (15 mg/ml)) was poured onto the separating gel. A comb was inserted and the gel allowed to set. On removing the comb, the gel was place in an electrophoresis tank and electrode buffer added.

Samples, as described in section 2.9 were loaded into the wells of the stacking gel and run through the stacking gel at a constant 60 V and through the separating gel at a constant 150 V until the dye front had run 8 cm. Electrophoresis was followed by staining.

2.11 STAINING OF POLYACRYLAMIDE GELS

2.11.1 Silver Staining For Lipopolysaccharides

Polyacrylamide gels were stained with silver by the method of Tsai & Frasch (1982), modified by Hancock & Poxton (1988). The following solutions were used:

(a) Fixative consisted of 7% w/v acetic acid and 25% propan-2-ol.

(b) Oxidising solution consisted of 1.05 g periodic acid in 150 ml distilled water to which 4 ml of fixative was added.

(c) Ammonical silver nitrate solution consisted of 1.4 ml ammonia solution (SG 0.88), 21 ml of 0.36% w/v NaOH , 4 ml 19.4% w/v silver nitrate solution made up to 100 ml with distilled water.

(d) Developing solution consisted of 0.019% v/v formaldehyde solution containing 0.005% citric acid.

The gel was placed in fixative overnight followed by oxidation in freshly prepared periodic acid for 5 min. After hourly washing over a 4 h period in distilled water, fresh ammonical silver nitrate solution was added for 15 min. Following four 10 min washes in distilled water, gels were transferred to freshly made developing solution. Once the gel had developed to the required intensity, it was repeatedly washed in large volumes of distilled water. The gel was stored in the dark until photographed. (All steps were with gentle agitation following fixation).

2.11.2 Coomassie Blue Staining For Proteins

The Coomassie blue staining method described in Hancock & Poxton (1988) was used. The following solutions made up in distilled water were used:

Solution 1- 2.5% v/v propan-2-ol, 10% v/v acetic acid and 0.05% w/v Coomassie brilliant blue R-250.

Solution 2- 10% v/v propan-2-ol, 10% v/v acetic acid and 0.0005% w/v Coomassie blue.

Solution 3- 10% v/v acetic acid and 0.0025 % w/v Coomassie blue.

Solution 4- 40% w/v methanol and 10% v/v acetic acid.

Solution 5- 10% v/v acetic acid.

The gel was placed in solution 1 overnight and sequentially placed in solutions 2-5 for 60 min at room temperature with gentle agitation throughout.

2.12 CHEMICAL ANALYSIS OF LIPOPOLYSACCHARIDES AND CAPSULAR POLYSACCHARIDES

2.12.1 Estimation Of Organic Phosphorus

The phosphorus content was measured by the method of Chen *et al* (1956). Solutions (in duplicate) containing up to 10 μg of phosphorus were reduced to dryness by heating on a micro Kjeldahl rack. To dry samples, 100 μl digestion mixture (concentrated H_2SO_4 and 60% perchloric acid mixed 3:2 v/v) was added and heated to boiling on the same rack and refluxed for 20 min. After cooling 8 ml of phosphate reagent (2% w/v ascorbic acid in 0.6 M H_2SO_4 containing 0.5% w/v ammonium molybdate) was added to each tube, mixed and incubated at 37°C for 90 min. The absorbance was read at 820 nm against a reagent blank. Phosphorus content was calculated relative to a potassium orthophosphate standard.

2.12.2 Estimation Of Carbohydrates

Neutral sugars were quantified using the colourimetric method described by Dubois *et al* (1956). Duplicate samples containing between 10-100 μg of carbohydrate were made up to 500 μl with PF-H_2O and 500 μl 5% w/v phenol solution was added. Concentrated H_2SO_4 (2.5 ml) was pipetted directly onto the samples so that mixing was instantaneous. After cooling for 30 min, the absorbance was read at 490 nm against a reagent blank. Carbohydrate content was calculated relative to a glucose standard.

2.12.3 Estimation Of KDO

Bacteroides LPSs were dephosphorylated by hydrofluoric acid as described by Beckmann *et al* (1989) and the KDO content of all LPSs determined by the use of a modified thiobarbiturate assay (Hancock & Poxton, 1988).

Approximately 5 mg of dry *Bacteroides* LPS was suspended in 300 µl of aqueous 50% hydrofluoric acid and sealed in polypropylene tubes at 4°C for 48 h. Volatile material was removed at room temperature under a stream of N_2. The residue was dissolved in 300 µl PF-H_2O and again dried under a stream of N_2. The dry residue was suspended in 1 ml PF-H_2O and kept at -20°C until required for further analysis.

Duplicate LPS (40 µl of a 5 mg/ml solution) samples were mixed with 1 ml of 0.125 M H_2SO_4 and heated to 100°C for 8 min. Upon cooling, 0.5 ml of acid hydrolysate was mixed with 0.25 ml of 25 mM periodic acid in 62.5 mM H_2SO_4 and incubated at 37°C for 30 min. After cooling, 0.25 ml of 2% w/v sodium arsenite in 0.5 M HCl was added and mixed until the brown iodine colour disappeared. Next, 0.25 ml 0.6% w/v thiobarbituric acid (adjusted to pH 9 with NaOH) was added and heated to 100°C for 7.5 min. While the reaction mixture was still hot, 1 ml of dimethylsulphoxide was added to all tubes and then allowed to cool. The absorbance was read at 548 nm against a reagent blank. KDO content was calculated relative to a standard of a known concentration.

2.12.4 Estimation Of Proteins

Protein was measured by the Folin Assay (Lowry *et al* 1951). Varying amounts of duplicate test samples were made up to 0.4 ml with PF-H_2O and 1.2 ml 12.5% w/v Na_2CO_3 and 0.2 ml 0.1% $CuSO_4.5H_2O$ added and mixed. After standing for 1 h at room temperature, the Folin reagent was diluted 1:3 and 0.2 ml added to each tube.

After standing at room temperature for 25 min the absorbance was read against a reagent blank. Protein content was calculated relative to a 2 mg/ml bovine serum albumin (BSA) standard included in the assay.

2.12.5 Gas Liquid Chromatography For Fatty Acids

Approximately 1 mg of LPS and 1 ml 0.5 M HCl in methanol were mixed and sealed in a glass tube before heating for 2 h at 65°C. Upon cooling, 2 ml of PF-H_2O and 4 ml ether were added, mixed and the phases allowed to separate. The upper ether layer was removed and dried in a rotary evaporator. Dry material was dissolved in 200 µl chloroform and transferred to a gas liquid chromatography (GLC) vial. Samples were stored at -20°C until analysis.

Methyl esters of long chain fatty acids were analysed on a Perkin-Elmer 8410 gas chromatograph fitted with an FID and a split/splitless injector. The split ratio was set at 100:1 and 1 µl samples were injected onto a BPI fused silica capillary column, 25 m x 0.22 mm ID and film thickness 0.25 µm (S.G.E.), with the linear velocity of the helium carrier gas set to 21.9 cm per second. The initial temperature of 150°C was held for 4 min before rising at 4°C per min to 250°C and held for 2 min. Bacterial acid methyl esters CP mix (Supelco (Supelchem UK Ltd), Saffron Walden, England) was used as a standard. Peak identity was confirmed by co-injection of the test materials and standard. Approximate molar ratios were calculated by comparison of peak areas.

2.13 LIGHT MICROSCOPY FOR CAPSULES

The presence of capsules was determined by light microscopy following Indian Ink staining. Equal volumes of an overnight culture of bacteria was mixed with Indian Ink

(Windsor & Newton) on a microscope slide, a coverslip added and firm pressure applied. Slides were viewed under x 40 bright field magnification.

2.14 PERCOLL GRADIENT CENTRIFUGATION

Percoll (Pharmacia) was supplied sterile. A stock solution, iso-osmotic with saline, was prepared by diluting with 1.5 M NaCl in a ratio of 9:1. The pH of this solution was adjusted to pH 7 with 1 M HCl. Solutions of 20, 40, 60 and 80% Percoll were prepared by further dilution with 0.15 M NaCl. A 1 ml volume of each of these solutions was layered carefully into a test tube to produce a step gradient with 80% Percoll at the bottom and 20% Percoll at the top. A broth culture of the test organism (1 ml) was applied to the top of the 20% layer and the gradient centrifuged at 2,600 g for 20 min in a bench centrifuge (Heraeus Christ).

2.15 MOUSE LETHALITY - GALACTOSAMINE (D-GAL N) MODEL

D-gal N (12 mg/mouse) was administered to groups of three, 6-8 week old C57 black male mice intraperitoneally just prior to administration of varying doses of LPS intraperitoneally from *Bacteroides* or *E. coli* species. Survival was recorded up to 24 h. Animal experiments were performed in accordance with Home Office guidelines. All animal manipulations were performed by the staff of the Animal House, University Medical School, Edinburgh.

2.16 LIMULUS AMOEBOCYTE ASSAY (LAL)

Stock LPS samples were diluted in PF-H_2O (Blood Transfusion Service, Protein Fractionation Centre, Edinburgh) to ranges of between 50 ng/ml and 0.5 ng/ml. Each sample (50 µl) was added in duplicate to wells of flat-bottomed microtitre plates (Griener, Cam, Dursley, England). Chromogenic LAL reagent (Coatest Endotoxin, Chromogenix, Sweden) was added to each well through a transfer plate to ensure that

each well received the LAL reagent at the same time. To minimise error through evaporation the outer wells of the plate were omitted. The plate was read kinetically every 19 s at 405 nm (reference background 650 nm) for 90 min in a Thermomax plate reader (Molecular Devices) at 37°C. The replicate mean onset time for test samples was standardised against an endotoxin of known potency *E. coli* O111:B4 (Coatest Kit Endotoxin Standard, Chromogenix).

2.16.1 LAL Assay Characteristics

Detection Level and Sensitivity

A log-log curve (Equation: log (y)= A + B*log (x)) was fitted to the standard dilutions and a correlation coefficient of greater than 0.99 was always achieved. Minimum detectable dose of endotoxin was 0.045 IU/ml (3.125 pg/ml of *E. coli* 0111.B4 LPS).

Specificity

The LAL gelation cascade is known to be triggered by primarily LPS and β-(1,3)-D-glucans. These glucans are found in the cell walls of fungi, yeast and algae.

Intra-Assay Precision

Samples of known endotoxin concentration were assayed in replicates of 10 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean IU/ml	10.2	55.6	75.4
Standard Deviation	1.2	4.9	6.3

Inter-Assay Precision

Samples were assayed 10 times in 5 different assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean pg/ml	9.5	52.3	77.9
Standard Deviation	1.5	4.6	6.8

2.17 LIPOPOLYSACCHARIDE AND CAPSULAR POLYSACCHARIDE INDUCED STIMULATION OF CYTOKINES

2.17.1 Stimulation Of Human Peripheral Mononuclear Leukocytes

Mononuclear leukocytes (MNL: approximately 30% monocytes) from freshly collected human buffy coats (obtained from the Blood Transfusion Service, Edinburgh) were prepared by sedimentation on lymphocyte separation medium (ICN Flow, Thame, England, UK). Cells were washed twice in RPMI-1640 and adjusted to 8 x 10^6 cells/ml and cultured in RPMI-1640 (Blood Transfusion Service, Edinburgh) supplemented with penicillin (100 U/ml) (Gibco), streptomycin (100 μg/ml) (Gibco), 1 mM L-glutamine (Gibco) and 10% foetal calf serum (FCS) (Gibco) in the absence and presence of varying nanogram concentrations of LPS or capsular polysaccharides (CP) at 37°C, 5% CO_2. Culture supernates were collected after 4 h or at one or two hourly intervals for time course experiments and stored at -20°C for TNF, IL-8, IL-6 or p55 sTNFr content determination.

2.17.2 Stimulation Of THP-1 Cells With And Without Enhancement For CD14

THP-1 cells (a human monocyte-macrophage cell line) were a kind gift from Dr David Morrison, University of Kansas Medical Centre, Kansas City, USA. Cells were not screened for mycoplasma as such a checking procedure has yet to be implemented in the Tissue Culture Laboratory, Medical School, Edinburgh. Cells were

retrospectively screened for mycoplasma contamination by Ciara Ryan and found to be clear. Cells were grown routinely in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM L-glutamine and 10% FCS at 37°C, 5% CO_2. Cells were subcultured once weekly by splitting 1:20. Cells were not passaged longer than three months. After this time, stock cultures from liquid nitrogen stores were used. Culture medium was routinely checked for bacterial contamination by testing medium on both aerobic and anaerobic blood agar plates.

To induce expression of CD14, cultures were grown in the presence of 0.1 μM 1,25-dihydroxy vitamin D3 (Sigma) for 72 h. Cells were harvested and washed once in RPMI-1640. Cells (2 x 10^6 cells/ml) were cultured in RPMI-1640 supplemented as before in the absence or presence of varying nanogram concentrations of LPS. Culture supernates were collected after 4 h or at two-hourly intervals for time course experiments and stored at -20°C for TNF, IL-8, IL-6 or p55 sTNFr content determination.

2.17.3 Stimulation Of Peritoneal Macrophages From LPS Responder And Non-Responder Mice

The LPS non-responder mice C3H/HeJ were obtained from Harlan Olac, Germany. The LPS responder mice C3H/HeN were obtained form Harlan Olac, England, UK. Sodium thioglycollate (1 ml of 10% w/v in distilled water) was injected intraperitoneally to each mouse four days prior to collection of peritoneal macrophages. To collect the macrophages (NB. this cell preparation will contain neutrophils and lymphocytes as well as macrophages), the mice were killed by cervical dislocation and the peritoneal cavity washed several times with 5 ml quantities of RPMI-1640. After washing the cells once in RPMI-1640, cells were resuspended to 2 x 10^6 cells/ml in RPMI-1640 supplemented as before in the presence or absence of

varying nanogram concentrations of LPS. Culture supernates were collected after 4 h or at two-hourly intervals for time course experiments and stored at -20°C for TNF content determination.

2.18 INHIBITION OF CYTOKINE PRODUCTION

2.18.1 Inhibition By Anti-CD14 Monoclonal Antibody

The anti-CD14 mouse anti-human IgG monoclonal antibody (mAb) was obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Scotland. The CD14 mAb was serially diluted 1:2 in RPMI-1640 and added to cell preparations (as described in section 2.17) so that the starting concentration was 75 μg/ml. Cell preparations and mAb were preincubated for 30 min at 37°C, 5% CO_2 before adding an appropriate dilution of LPS and reincubating as before. Culture supernates were harvested at 4 h and stored at -20°C for determination of TNF, IL-6 and IL-8 content.

2.18.2 Inhibition By Anti-*Bacteroides* Monoclonal Antibodies

Cell lines raised against *B. fragilis* NCTC 9343, producing monoclonal antibodies were obtained from Dr Sheila Patrick, Queen's University of Belfast and grown routinely in RPMI-1640 supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 1 mM glutamine and sodium pyruvate 1.1 g/l (Gibco). Once cells had grown to maximum density, cells were centrifuged and the supernates collected, aliquoted and stored at -20°C until required. See Table 2 below for details. Supernates were serially diluted 1:2 in RPMI-1640 and added to cell preparations (as described in section 2.17). Cell preparations and mAb supernates were preincubated for 30 min at 37°C, 5% CO_2 before adding an appropriate dilution of LPS and

reincubating as before. Culture supernates were harvested at 4 h and stored at -20°C for determination of TNF and IL-8 content.

Table 2: Monoclonal Antibodies Raised Against *B. fragilis* NCTC 9343

Cell Line	Active Against	Isotype
3C8	H mwt, O Ag	IgG 2b
3D7	H mwt	IgG 1
4CS	H mwt	IgG 1
1A4	H mwt	?
6G3	No H mwt only O Ag	IgG 2b
5A12	Complete LPS ladder	IgG 1

The above table shows information on each cell line obtained from Dr Sheila Patrick, Queen's University of Belfast. Cell lines had been raised and tested by immunoblotting against *B. fragilis* NCTC 9343. Where H mwt = high molecular weight LPS material, O Ag = O antigen LPS material, ? = not known.

2.18.3 Inhibition By Anti-TNF Monoclonal Antibody

Cell culture supernates derived from human mononuclear leukocytes as described in section 2.17.1 were incubated with various dilutions of an anti-TNF mAb (Genzyme, Hartfield, England) in RPMI-1640 for 1 h at 37°C, 5% CO_2 prior to addition of the culture supernate to the L929 cells as described in section 2.19 below.

2.19 DETERMINATION OF TNF CONTENT BY L929 BIOASSAY

The L929 mouse fibroblast cell line which is sensitive to TNF, was cultured in growth medium: MEM (Sigma) containing 5% FCS supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 1 mM L-glutamine, and maintained by splitting 1:10 twice weekly. Cells were subcultured once weekly by splitting 1:20. Cells were not

passaged longer than three months. After this time, stock cultures from liquid nitrogen stores were used. Cells were not screened for mycoplasma as such a checking procedure has yet to be implemented in the Tissue Culture Laboratory, Medical School, Edinburgh. Culture medium was routinely checked for bacterial contamination by testing medium on both aerobic and anaerobic blood agar plates.

Cells were dislodged by 0.005% trypsin/0.02% EDTA to avoid cell clumping, washed and resuspended in growth medium to 3 x 10^5 cells/ml. Cells were dispensed in flat-bottomed microplates (Greiner) at 100 µl/well and incubated at 37°C, 5% CO_2 for 20 h. The growth medium was then aspirated, discarded and replaced with 100 µl/well of assay medium: MEM containing 5% FCS supplemented with 1 mM glutamine and 2 µ g/ml actinomycin-D (to stop further cell growth without killing the cells). To experimental wells, 100 µl of test supernate which had been diluted 1:5 in assay medium was added. A standard of recombinant TNF (National Institute for Biological Standards and Control, UK) diluted serially 1:5 at a starting concentration of 1000 IU/ml and wells without TNF were included. Plates were covered and incubated at 37°C, 5% CO_2 for 24 h. The medium was then discarded and replaced with 100 µl/well of filtered (0.22 µm) crystal violet solution (0.5% crystal violet in 20% (v/v) methanol in distilled water) which stains surviving cells. After 2 min the plates were washed vigorously under tap water and dried. The crystals were dissolved by addition of 100 µl of 20% (v/v) acetic acid to all wells and the plate read at 585 nm on a Vmax plate reader (Molecular Devices). The content of TNF was calculated relative to the standard curve.

2.19.1 TNF Bioassay Characteristics

Detection Level and Sensitivity

A 4-parameter curve (Equation y=(A-D)/(1+ (X/C)^B-D) was fitted to the standard dilutions. This gave a very good fit with correlation coeffecients greater than 0.990. Using only the linear part of the curve TNF concentrations ranging from 0.5 to 300 IU/ml could be accurately measured.

Specificity

Anti-TNF mAb completely inhibited cytotoxicity to L929 cells in this assay system. (See section 3.10.4, Table 16 in Results)

Intra-Assay Precision

Samples of known TNF concentration were assayed in replicates of 10 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean IU/ml	5.6	75.2	150.9
Standard Deviation	0.9	6.9	9.6

Inter-Assay Precision

Samples were assayed 10 times in 5 different assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean pg/ml	6.3	80.6	153.4
Standard Deviation	2.9	9.2	12.1

2.20 INTERLEUKIN-8 RADIO IMMUNOASSAY

Test supernates were diluted 1:5 and 1:10 in duplicate in assay buffer (16% w/v Na_2HPO_4, 4% w/v KH_2PO_4, 0.1% v/v Triton X-100, 0.3% v/v BSA (Boroseral) and 0.05% w/v NaN_3 in distilled water pH 7.4) and 100 µl added to each assay tube. A standard of human recombinant IL-8 (National Institute for Biological Standards and Control, UK) was diluted serially 1:10 at a starting concentration of 5 mg/ml and controls of total count and non-specific binding were also included in the assay. The IL-8 antibody (a rabbit anti-human IgG obtained from Dr R.W. Kelley, Centre for Reproductive Biology, Edinburgh) was diluted 1:30,000 in assay buffer and 25 µl added to each tube except total count and non-specific binding controls. Tubes were left overnight at 4°C before adding 25 µl of tracer (IL-8 antibody bound to ^{125}I, obtained from Dr R.W. Kelley) which had been adjusted to 15,000 cpm. Tubes were left again overnight at 4°C. A second antibody solution consisting of donkey anti-rabbit IgG (SAPU) diluted 1:20 and normal rabbit serum (SAPU) diluted 1:200 was made up in assay buffer containing 4% PEG. Second antibody solution (200 µl) was added to each tube and left for 1 h at 4°C. The tubes were then spun at 3,200 g for 20 min at 4°C, the supernates aspirated and counted in a gamma counter (Packard Instruments). The IL-8 content of each test sample was calculated relative to the standard curve.

2.21 IL-8 Radio-Immunoassay Characteristics

Detection Level and Sensitivity

A 4-parameter curve (Equation y=(A-D)/(1+ (X/C)^B-D) was fitted to the standard dilutions. This gave a very good fit with correlation coeffeicents greater than 0.990.

Using only the linear part of the curve IL-8 concentrations ranging from 10pg/ml to 40000pg/ml could be accurately measured.

Specificity

The IL-8 antibody used in this assay is not known to cross react with IL-1, IL-2, IL-6 and IL-10.

Intra-Assay Precision

Intra assay variation of < 5% has been noted.

Inter-Assay Precision

Inter-assay variation of <13% has been noted.

2.21 INTERLEUKIN-6 ELISA

Interleukin-6 was measured using the commercially available kit Cytoscreen (Biosource International, Camarillo, CA, USA). All solutions described below were supplied ready to use in the kit and exact information on each solution was not available. In brief, to IL-6 antibody-coated wells 100 µl of standard diluent was added to the blanks, 50 µl of the standard diluent was added to the zero wells, and 50 µl of standards/culture supernates were added. Biotinylated anti-IL-6 antibody (50 µl) solution was added to each well except the blank wells, the plate covered and incubated for 2 h at 37°C. Plates were washed thoroughly three times with wash buffer before adding 100 µl of Streptavidin-HRP working conjugate to all wells. Plates were covered and incubated at room temperature for 1 h before washing thoroughly four times in wash buffer. Stabilised Chromogen (100 µl) was added to each well and incubated in the dark for 20 min before adding 100 µl of Stop Solution

to each well. The plate was read at 450 nm on a Vmax plate reader (Molecular Devices) and the content of IL-6 calculated relative to the standard curve.

2.21.1 IL-6 ELISA Characteristics

Detection Level and Sensitivity

Minimum detectable dose of IL-6 was less than 2 pg/ml.

Specificity

Buffered solutions of a panel of substances were assayed in IL-6 kit at known concentrations. No cross-reactivity was indicated in any case. Percentage cross-reactivity is estimated to be less than 0.005% in each case of IL-1β, IL-2, IL-3, IL-7, IL-8 and TNF-α.

Intra-Assay Precision

Samples of known IL-6 concentration were assayed in replicates of 10 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean pg/ml	103.3	144.5	228.5
Standard Deviation	6.9	9.9	8.3

Inter-Assay Precision

Samples were assayed 10 times in 5 different assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean pg/ml	65.9	124.3	250.1
Standard Deviation	3.0	10.4	10.0

2.22 PROLIFERATION ASSAY

Spleen or lymph nodes were aseptically removed from C3H/HeN mice and placed in RPMI-1640. Tissues were homogenised gently to make a single cell suspension and filtered through sterile gauze. Cells were washed twice with RPMI-1640 and resuspended to 2 x 10^6 cells/ml in RPMI-1640 containing 5% FCS for the assay and 2 x 10^7 cells/ml and treated as described below.

Preparation of T cells: Aliquots of 1 ml of 2 x 10^7 cells/ml were added to the top of equilibrated nylon wool columns. The cells were allowed to run into the nylon wool column before incubating at 37°C, 5% CO_2 for 45 min. T cells were eluted by running 10-15 ml of RPMI-1640 through the column. Cells were washed once in RPMI-1640 and resuspended to 2 x 10^6 cells/ml in RPMI-1640 containing 5% FCS for the assay.

Preparation of B cells: Aliquots of 2 x 10^7 cells/ml were incubated in tissue culture flasks at 37°C, 5% CO_2 for 1 h to remove the monocytes/macrophages. An anti-CD3 mAb (Cedarlane, VH Bio Ltd, Gosforth, England) was added at a 1:40 dilution and incubated for 40 min at 4°C. Cells were washed once in RPMI-1640 and resuspended to the original volume before 0.5 ml of complement (Guinea pig Haemo-10, Cedarlane) was added. After incubation at 37°C, 5% CO_2 for 30 min, cells were centrifuged gently to remove dead cells and the viable cells adjusted to 2 x 10^6 cells/ml in RPMI-1640 containing 5% FCS for the assay.

Assay: Cell suspension (100 µl) was added to an equal volume of an appropriate dilution of phytohaemagglutinin (PHA) (Sigma) or LPS in a 96 well plate in triplicate. Plates were covered and incubated at 37°C, 5% CO_2 for 48h, before adding 10 µCi of ^{3}H-thymidine (Amersham, England,UK) in 20 µl of RPMI-1640 to each well.

Plates were covered and incubated for a further 24 h at 37°C, 5% CO_2. Wells were washed with distilled water using an Automash 2000 washer (Dynatech) and deposited onto filter paper (Filter Paper for Cell Harvesters, ICN Flow). The filter paper was allowed to dry overnight before pushing out the filter paper discs and placing them into Ponyvial polyethylene vials (Packard). Hydrolumac scintillation fluid (1 ml) (Lumac LSC, Belgium) was added to each vial, capped and the beta emission measured in a Tricarb Liquid Scintillation Analyser 1900 CA (Packard).

2.23 COLLECTION OF HUMAN SERUM

From a healthy volunteer, 20 ml of blood was collected and decanted into a sterile universal. Blood was allowed to clot at 37°C for 30 min. Red blood cells were removed by centrifugation at 3,900 g and the serum removed. Serum was aliquoted in cryogenic tubes (Nunc, Intermed, Denmark) and stored at -70°C until required.

2.24 CH_{50} COMPLEMENT ASSAY

The effect of LPS on the CH_{50} (haemolytic complement value) of human serum was examined. Sheep red blood cells (SRBC) were firstly sensitised with an anti-SRBC antibody. Approximately 1 ml of SRBC (SAPU) were washed twice in PBS and the cells resuspended in PBS to 1 x 10^9 cells/ml. An equal volume of this cell suspension was mixed with a 1:1000 rabbit anti-SRBC antibody (SAPU) in PBS and incubated at 37°C for 30 min. After washing twice in PBS, the SRBC were resuspended in the original volume to give 1 x 10^9 cells/ml for use in the assay.

Tubes were set out as in the protocol below, with one set for each LPS type. Tubes were incubated at 37 °C for 45 min before adding 3 ml of ice cold PBS to each tube. After centrifuging at 200 g for 5 min to remove all cells, the absorbance of each supernate was measured at 541 nm.

Protocol: ± Constant concentration of each LPS (ng) in each tube per set. *Human serum collected as described in section 2.23 diluted 1:5 in PBS for use.

	Tube Number						
	1	2	3	4	5	6	7
PBS (ml)	1.1	1.05	1.0	0.9	0.8	1.2	1.2 H_2O
Serum (ml)*	0.1	0.15	0.2	0.3	0.4	0.0	0.0
Sensitised SRBC (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3

Assuming that tube 7 represents total lysis and tube 6 spontaneous lysis, percentage lysis was calculated for each tube. Taking y to be percentage lysis and x to be volume of serum used, a graph of log (y/100-y) against log x was plotted. The straight line has a slope if 1/n. The abscissa intercept of the line where log(y/100-y)=0 is the log dilution resulting in 50% lysis i.e. one CH_{50} unit.

2.25 FLOW CYTOMETRY

2.25.1 Analysis of CD14 Positive Cells

THP-1 cells (with and without enhancement for CD14) and human mononuclear leukocytes were examined by flow cytometry for CD14 distribution. Cells were grown as described in section 2.17 and resuspended in RPMI-1640 to approximately 3 x 10^6 cells/ml. Cells (100 µl) were added to 25 µl of normal rabbit serum (SAPU) and incubated at room temperature for 20 min. Cells were washed once in PBS, the supernate removed and replaced with 100 µl of various dilutions of an anti-CD14 mAb (SAPU, see section 2.18.1) and incubated at room temperature for 30 min. Cells were washed twice in PBS, the supernate removed and replaced with 100 µl of a

1:100 dilution of FITC-conjugated anti-mouse IgG (SAPU). After incubating for 30 min at room temperature, cells were washed twice in PBS, fixed in 200 µl of buffered paraformaldehyde (10% v/v paraformaldehyde, 10.7% w/v sodium cacodylate, 7.5% w/v NaCl adjusted to pH 7.2 with HCl) and stored at 4°C until required for analysis.

2.25.2 Analysis of B and T Cells From Mouse Spleens

Cells (spleen mix, B and T cells) prepared as described in section 2.22 were examined by flow cytometry to ensure homogeneous cell populations. Red blood cells were firstly lysed by addition of one part distilled H_2O to three parts PBS. Cells (100 µl) at 2 x 10^6 per ml were added in triplicate to V-bottomed microtitre plates (Griener). Cells were pelleted out and resuspended in residual buffer before adding 25 µl of normal rabbit serum. After incubation for 30 min on ice, cells were washed once in 100 µl in PBS before adding 10 µl of an anti-mouse CD3 mAb (SAPU). The incubation step was repeated and the cells washed three times in 100 µl of PBS before adding 100 µl of an anti-mouse FITC IgG (SAPU) that had been diluted 1:20. The incubation step was repeated and the cells washed four times in 100 µl of PBS. Buffered paraformaldehyde (100 µl) was added to each well and the sample stored at 4°C until analysis. As a control, cells without the first mAb and without any mAb treatment were also included.

Samples were analysed in an EPICS "C" (Coulter Electronics) flow cytometer. A total of 5000 cells were analysed. Background noise and clumps of cells were excluded by a gate on the forward angle light scatter. The percentage of cells exhibiting positive fluorescence was calculated with the EPICS "Stat Pack" Program.

2.26 COATING OF LPSs ONTO MICROTITRE PLATES

All LPSs (1 mg/ml) were mixed with polymyxin B (Sigma) (1 mg/ml) in a ratio of 1:1 in Reactivials (Pierce, Luton, England). Solutions were sonicated in short bursts for 30 s and stirred at room temperature for 1-2 h. The solutions were sonicated for a further 30 s and then dialysed for 18-20 h at 4°C against PF-H_2O in Spectra-Por dialysis membrane (mwt cut off 2,000) (Spectrum, Houston, USA). Following dialysis, solutions were diluted 1:50 in coating buffer (0.05 M carbonate/bicarbonate pH 9.6 containing 0.02% w/v NaN_3) and plates (Greiner Removastrip) were coated at 100 µl/well. Plates were sealed and incubated overnight at room temperature before washing four times in wash buffer (pyrogen-free PBS containing 0.05% Tween 20 and 0.02% w/v NaN_3). Plates were post coated (pyrogen free PBS containing 5% w/v BSA and 0.02% w/v NaN_3) at 100 µl/well. Plates were sealed and incubated overnight at room temperature before washing four times in wash buffer and rinsing once in PF-H_2O. Plates were stored at -20°C until required.

2.27 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Bacteroides mAb supernates were diluted appropriately in dilution buffer (PF-PBS containing 0.05% w/v Tween 20, 0.5% w/v BSA, 4% w/v PEG-6000 and 0.02% w/v NaN_3). A positive human normal serum control was diluted 1:200 in dilution buffer and 100 µl of each dilution in duplicate added per well. Plates were sealed and incubated for 90 min at 37°C before washing four times in wash buffer. An anti-mouse IgG alkaline phosphatase conjugate (Seralab, Crawley Down, England) was diluted 1:1000 in dilution buffer and 100 µl added to the *Bacteroides* mAb supernate wells. An anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in dilution buffer and 100 µl added to the positive human normal serum control wells. Plates were sealed and incubated for a further 90 min at 37°C before washing four times in wash buffer. Alkaline phosphatase substrate tablets 104 (Sigma) were diluted

to 1 mg/ml in substrate solvent (0.05 M sodium carbonate buffer pH 9.8 containing 1 mM $MgCl_2$) and 100 µl added to all wells. Plates were incubated for 15-45 min at room temperature and then read at 405 nm (background 620 nm) in an Anthos reader 2001 (Labtec).

Two negative controls were also included. First was a well for each mAb dilution which was not coated in antigen but the primary and secondary antibody still applied. The second was wells coated in antigen but the primary antibody replaced by pure diluent.

2.28 SANDWICH ELISA FOR QUANTITATION OF p55 SOLUBLE TNF RECEPTORS

The reagents and method for the sandwich ELISA for the quantitation of p55 soluble TNF receptors (sTNFr) were kindly donated by Celltech Ltd, Slough, UK.

Anti p55 soluble TNF receptor antibody 5R13 was diluted to 5 µg/ml in coating buffer (20 mM BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 2-(bis(2-hydroxyethyl) amino) ethanesulfonic acid), pH 6) and 100 µl dispensed into each well (Nunc Maxisorb immunostrip flat bottomed plates). Plates were sealed and incubated overnight at room temperature before washing twice in blocking buffer (Dulbeccos PBS containing 1% w/v BSA) and then twice in glazing buffer (5% w/v lactose and 0.1% w/v BSA in distilled water). Plates were allowed to dry, sealed in a foil pouches containing a silica gel sachet and stored at 4°C until required.

Assay diluent (Dulbeccos PBS containing 1% w/v BSA) (100 µl) was dispensed into each well. Supernates obtained as described in sections 2.17.1 and 2.17.2 were added (10 µl) in duplicate to appropriate wells. p55 sTNFr standards and interassay

controls were also added in duplicate (10 µl) to appropriate wells. Plates were sealed and incubated with agitation for 1 h at room temperature before washing twice with PBS. 5R5-Biotin solution was diluted in assay diluent to 1 µg/ml and 100 µl dispensed into each well. Plates were sealed and incubated with agitation for 30 min at room temperature before washing twice in PBS. Streptavidin-HRP conjugate (Sigma) was diluted in assay diluent to 1 µg/ml and 100 µl dispensed into each well. Plates were sealed and incubated with agitation for 30 min at room temperature before washing four times with PBS. TMB substrate (tetramethylbenzidine dihydrochloride) (Sigma) was prepared in 0.05 M phosphate-citrate buffer pH 5 containing 0.006% v/v H_2O_2 (added just prior to use) and 100 µl dispensed into each well. Plates were sealed and incubated with agitation for 10-20 min at room temperature. Plates were read at 650 nm (background reference 405 nm) on a Vmax plate reader (Molecular Devices) and p55 sTNFr concentration of each sample calculated relative to the standard curve.

2.29 PRECAUTIONS AGAINST ENDOTOXIN CONTAMINATION

As the majority of assays and techniques in this thesis can detect and are sensitive to picogram amounts of endotoxin it was very important not to introduce any contaminating endotoxin into test samples and assay systems. The following precautions were therefore undertaken:

-Use of PF-H_2O where appropriate (Milli-Q pyrogen free unless indicated).

-Use of pyrogen free (PF) pipette tips where appropriate.

-Use of PF tissue culture plates, plastic wear, tubes, troughs etc.

-All reusable glassware was depyrogenated by heating to 250°C for 3 h.

-All LPS samples aliquoted to avoid freeze/thaw cycle.

-All media, buffers, pipettes and pipette tips supplied sterile.

-Manipulations using tissue culture, LAL assay and LPS samples carried out in a Class II safety cabinet.

2.30 STATISTICAL METHODS EMPLOYED

Certain experiments were analysed by statistics (see sections 3.10.6 and 3.10.11). The student t-test and Mann Whitney test to compare medians were used and in another instance mean TNF values were normalised to the value for *E. coli* O18K$^-$ LPS which was given the arbitrary value of 100%. The inter-assay variation between different buffycoats preparations was high (ranging from 5% to 45%, data not shown) and for this reason results from MNL experiments were presented as the mean of a least two experiments. All results depict trends which have confirmed on at least two occasions. For consistency, other experiments were also presented in this manner. Error bars were omitted from graphs to allow for a clearer representation of results.

Results

NOTE

Unless otherwise indicated in the results or discussion, LPS samples refer to protein decontaminated native phenol-water (PW) extracted LPS, which have been prepared from bacteria grown in PPY medium for *Bacteroides* species and NB for *E. coli* O18K$^-$. *E. coli* O18K$^-$ was aerobically grown unless otherwise indicated. Serum is included in all experiments unless indicated. In every stimulation experiment where used, 8 x 10^6 cells/ml of human MNL, 2 x 10^6 cells/ml of THP-1 cells with and without enhancement for CD14 and 2 x 10^6 cells/ml of peritoneal macrophages from LPS responder and non-responder mice were used. Representation of results is explained in the Methods section 2.30.

3.1 THE EFFECT OF GROWTH MEDIUM ON LPS CHEMOTYPE

The effect of growth medium on LPS chemotype was examined in 25 strains of *Bacteroides*. Bacteria were grown in PPY medium, VT medium or VT medium containing 50% HISS and their LPS chemotype compared by proteinase K extracts on silver stained PAGE (Fig 1 A-D). The *E. coli* O18K$^-$ LPS control had been grown in nutrient broth (NB). No clear differences in silver-stained profiles were seen between bacteria grown in different growth medium. All *B. vulgatus* strains failed to grow in VT medium. Several *Bacteroides* species showed characteristic silver-stain profiles. A banding pattern typical of smooth LPS was seen for all *B. vulgatus* species (Fig 1, gel A tracks 14-19, gel B tracks 2, 4) and the *E. coli* O18K$^-$ control. Most of the *Bacteroides* species showed silver-stain profiles of low molecular mass material only.

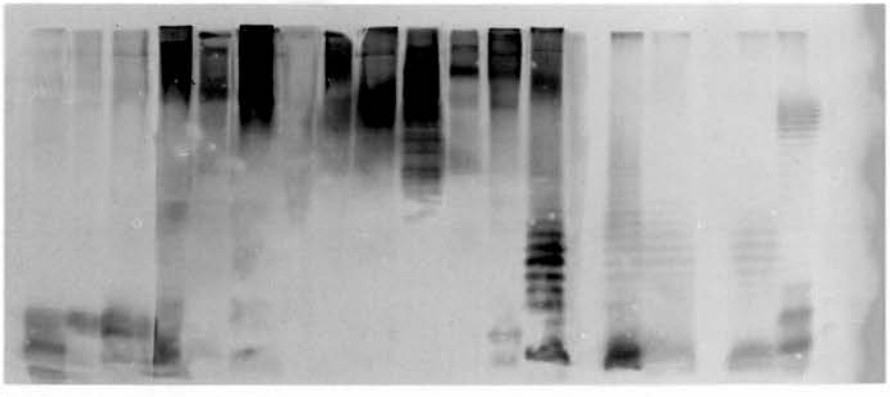
3.2 INVESTIGATION INTO SUB-POPULATIONS OF *BACTEROIDES* SPECIES

Sub-populations of *Bacteroides* were examined in 12 strains (see Table 1 Materials & Methods). Bacteria were grown in VT medium and sub-populations separated on Percoll density gradients (Fig 2). Bacteria from the bands were removed with a sterile syringe and stained for capsules with indian ink (Fig 3) or sub-cultured in VT medium and their LPS chemotype within sub-populations examined by comparing Proteinase K extracts on silver-stained gels (Fig 4 A, B).

After centrifugation of cultures in defined medium on a Percoll density gradient (Fig 2), cells with large capsules remained on top of the 20% layer and non-capsulate cells were concentrated at the 80% layer. Most strains tested consisted of mixtures of small/large/non-capsulate bacteria except for *B. fragilis* MPRL 1504, which consisted of 100% large capsulate bacteria. All *B. vulgatus* strains exhibited the same Percoll

Fig 1: Silver stain profiles of Proteinase K extracts of 24 *Bacteroides* species grown in either PPY[a], VT [b] or VT + 50% HISS[c] medium

Gel A

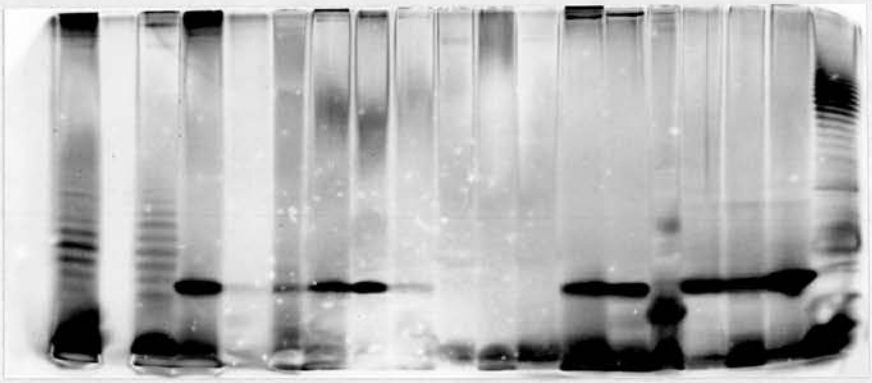


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Gel A: Track 1= sample buffer, 2= *B. fragilis* NCTC 9343[a], 3= *B. fragilis* NCTC 9343[b], 4= *B. fragilis* NCTC 9343[c], 5= *B. fragilis* MPRL 1504[a], 6= *B. fragilis* MPRL 1504[b], 7= *B. fragilis* MPRL 1504[c], 8= *B. fragilis* MPRL 1652[a], 9= *B. fragilis* MPRL 1652[b], 10= *B. fragilis* MPRL 1652[c], 11= *B. fragilis* MPRL 1978[a], 12= *B. fragilis* MPRL 1978[b], 13= *B. fragilis* MPRL 1978[c], 14= *B. vulgatus* MPRL 1726[a], 15= *B. vulgatus* MPRL 1726[b] NG , 16= *B. vulgatus* MPRL 1726[c], 17= *B. vulgatus* MPRL 1651[a], 18= *B. vulgatus* MPRL 1651[b] NG, 19= *B. vulgatus* MPRL 1651[c], 20= *E. coli* O18K$^-$.

Where *E. coli* O18K$^-$ control was grown in NB and NG = no growth.

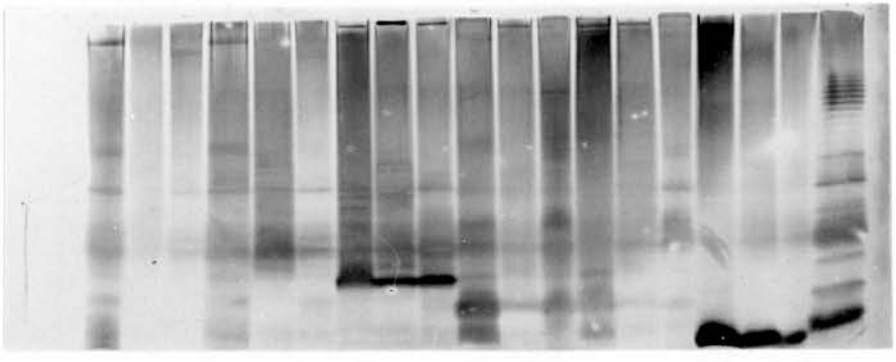
Gel B



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Gel B: Track 1= sample buffer, 2= *B. vulgatus* MPRL 1985[a], 3= *B. vulgatus* MPRL 1985[b] NG, 4= *B. vulgatus* MPRL 1985[c], 5= *B. thetaiotaomicron* MPRL 1720[a], 6= *B. thetaiotaomicron* MPRL 1720[b], 7= *B. thetaiotaomicron* MPRL 1720[c], 8= *B. thetaiotaomicron* MPRL 1959[a], 9= *B. thetaiotaomicron* MPRL 1959[b], 10= *B. thetaiotaomicron* MPRL 1959[c], 11= *B. merdae/distasonis* MPRL 1522[a], 12= *B. merdae/distasonis* MPRL 1522[b], 13= *B. merdae/distasonis* MPRL 1522[c], 14= *B. caccae* MPRL 1555[a], 15= *B. caccae* MPRL 1555[b], 16= *B. caccae* MPRL 1555[c], 17= *B. ovatus* MPRL 1709[a], 18= *B. ovatus* MPRL 1709[b], 19= *B. ovatus* MPRL 1709[c], 20= *E. coli* O18K$^-$.

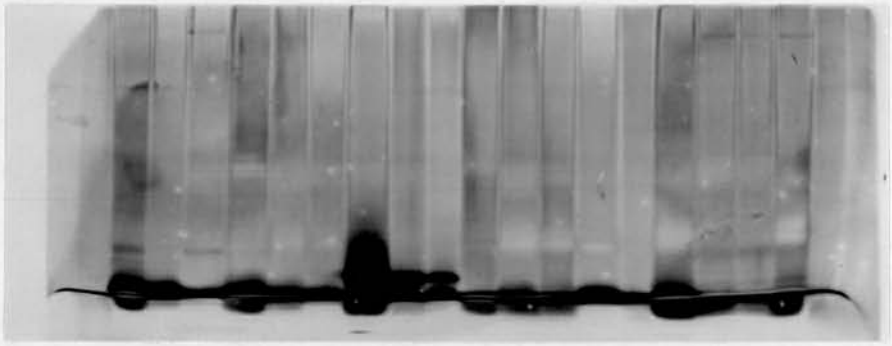
Gel C



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Gel C: Track 1= sample buffer, 2= *B. uniformis* MPRL 1721[a], 3= *B. uniformis* MPRL 1721[b], 4= *B. uniformis* MPRL 1721[c], 5= *B. uniformis* MPRL 1542[a], 6= *B. uniformis* MPRL 1542[b], 7= *B. uniformis* MPRL 1542[c], 8= *B. thetaiotaomicron* MPRL 1987[a], 9= *B. thetaiotaomicron* MPRL 1987[b], 10= *B. thetaiotaomicron* MPRL 1987[c], 11= *B. variabilis* MPRL 1742[a], 12= *B. variabilis* MPRL 1724[b], 13= *B. variabilis* MPRL 1724[c], 14= *B. variabilis* MPRL 2244[a], 15= *B. variabilis* MPRL 2244[b], 16= *B. variabilis* MPRL 2244[c], 17= *B. eggerthii* MPRL 1668[a], 18= *B. eggerthii* MPRL 1668[b], 19= *B. eggerthii* MPRL 1668[c], 20= *E. coli* O18K$^-$.

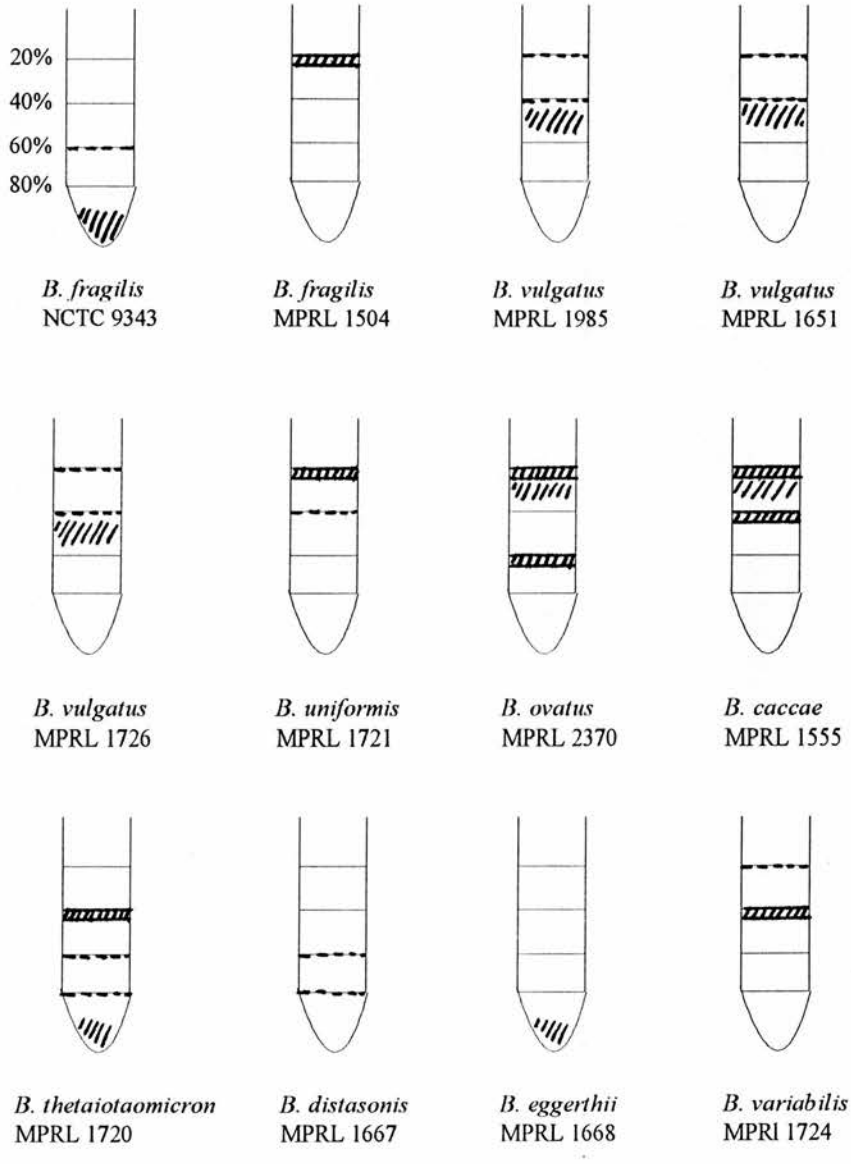
Gel D



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Gel D: Track 1= sample buffer, 2= *B. eggerthii* MPRL 1523[a], 3= *B. eggerthii* MPRL 1523[b], 4= *B. eggerthii* MPRL 1523[c], 5= *B. eggerthii* MPRL 1216[a], 6= *B. eggerthii* MPRL 1216[b], 7= *B. eggerthii* MPRL 1216[c], 8= *B. caccae* MPRL 2278[a], 9= *B. caccae* MPRL 2278[b], 10= *B. caccae* MPRL 2278[c], 11= *B. merdae* MPRL 2277[a], 12= *B. merdae* MPRL 2277[b], 13= *B. merdae* MPRL 2277[c], 14= *B. stercoris* MPRL 2276[a], 15= *B. stercoris* MPRL 2276[b], 16= *B. stercoris* MPRL 2276[c], 17= *B. distasonis* MPRL 1667[a], 18= *B. distasonis* MPRL 1667[b], 19= *B. distasonis* MPRL 1667[c]

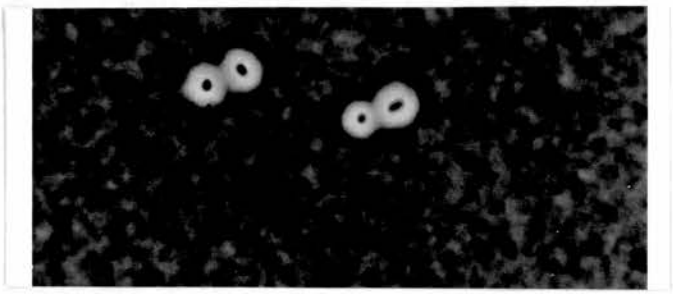
Fig 2: Percoll profiles of 12 strains of *Bacteroides* after growth in VT medium



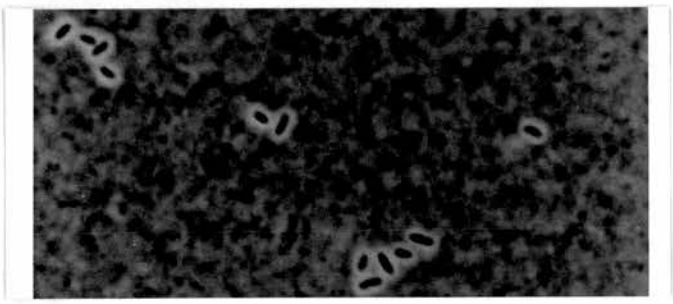
Where ---- faint band, ///// thick band and ////// clouding.

Fig 3: Typical Indian ink capsule stains from *B. fragilis* NCTC 9343 after growth in VT medium

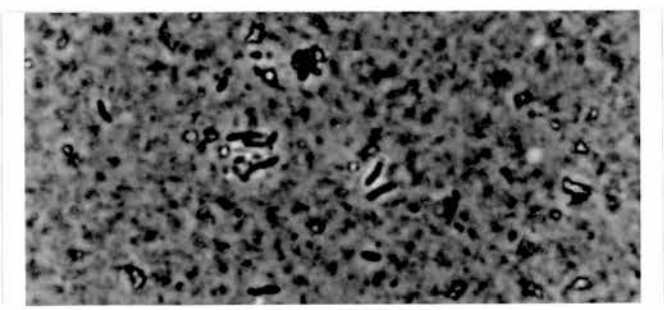
a) Large capsulate bacteria



b) Small capsulate bacteria



c) Non-capsulate bacteria



profile. No capsules were seen in *B. eggerthii* MPRL 1668 cultures. A typical indian ink capsule stain showing small/large/non-capsulate bacteria is shown in Fig 3 (a-c). No difference in LPS chemotype within sub-populations of *Bacteroides* species was seen in Proteinase K extracts (Fig 4 A, B). Sub-populations of bacteria were reconfirmed by Percoll gradients and found to consist of homogeneous populations of cells (data not shown).

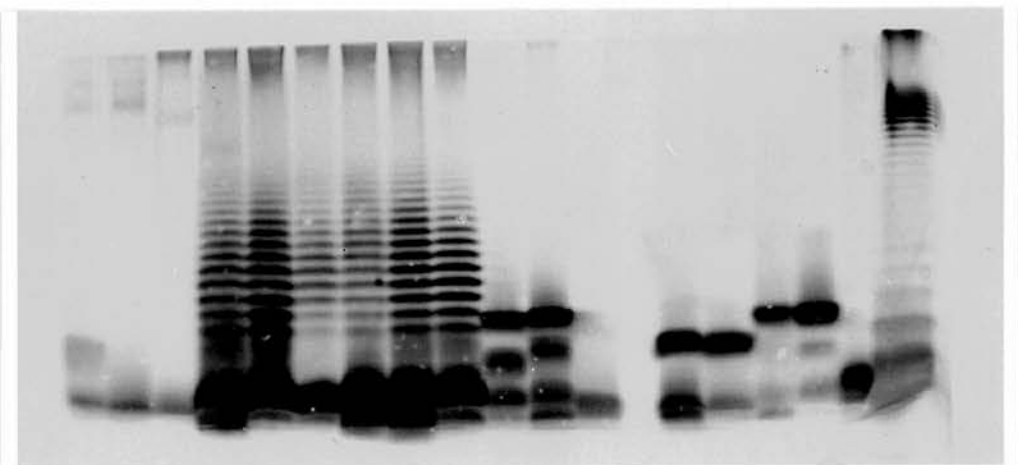
3.3 EFFECT OF DIFFERENT ENVIRONMENTAL ATMOSPHERES ON GROWTH OF *BACTEROIDES* SPECIES

As a precursor to large scale fermentations in the preparation of a LPS library, the growth of 10 strains of *Bacteroides* grown in PPY or VT medium were examined in three different environmental atmospheres: 10% CO_2 and 90% N_2; 10% CO_2 and 90% H_2; 100% N_2 (Table 1).

Each strain tested grew well in PPY media in all environmental atmospheres. Most strains grew poorly in VT medium, especially in an atmosphere of 100% N_2. From these results it was decided to use VT medium in one fermenter run and PPY medium in the remaining fermenter runs for the preparation of the LPS library (see section 3.4 below). The chosen environmental atmosphere was 100% N_2 as it does not require a catalyst and was easy to administer.

Fig 4: Silver stain profiles of Proteinase K extracts from sub-populations of *Bacteroides* strains separated by Percoll density centrifugation

Gel A



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Gel A: Track 1= sample buffer, 2= *B. fragilis* NCTC 9343 60%, 3= *B. fragilis* NCTC 9343 80%, 4= *B. fragilis* MPRL 1504 20%, 5= *B. vulgatus* MPRL 1726 20%, 6= *B. vulgatus* MPRL 1726 40%, 7= *B. vulgatus* MPRL 1651 20%, 8= *B. vulgatus* MPRL 1651 40%, 9= *B. vulgatus* MPRL 1985 20%, 10= *B. vulgatus* MPRL 1985 40%, 11= *B. caccae* MPRL 1555 20%, 12= *B. caccae* MPRL 1555 40%, 13= *B. uniformis* MPRL 1721 20%, 14= *B. uniformis* MPRL 1721 40%, 15= *B. variabilis* MPRL 1724 20%, 16= *B. variabilis* MPRL 1724 40%, 17= *B. ovatus* MPRL 2370 20%, 18= *B. ovatus* MPRL 2370 60%, 19= *B. eggerthii* MPRL 1668 80%, 20= *E. coli* O18K$^-$.

Gel B

1 2 3 4 5 6

Gel B: Track 1= sample buffer, 2= *B. thetaiotaomicron* MPRL 1720 40%, 3= *B. thetaiotaomicron* MPRL 1720 60%, 4= *B. thetaiotaomicron* MPRL 1720 80%, 5= *B. distasonis* MPRL 1667 60%, 6= *B. distasonis* MPRL 1667 80%.

Table 1: Growth of 10 *Bacteroides* species in three different environmental atmospheres

Species /Strain number	Growth Media	Growth in Environmental Atmospheres		
		10% CO_2 90% H_2	10% CO_2 90% N_2	100% N_2
B. fragilis MPRL 1504	PPY	+++	+++	+++
	VT	+	+	+
B. fragilis NCTC 9343	PPY	+++	+++	+++
	VT	++	++	+
B. vulgatus MPRL 1985	PPY	+++	+++	+++
	VT	++	+	+
B. thetaiotaomicron MPRL 1720	PPY	+++	+++	+++
	VT	++	+	-
B. caccae MPRL 1555	PPY	+++	+++	+++
	VT	++	++	-
B. uniformis MPRL 1721	PPY	+++	+++	+++
	VT	-	++	-
B. ovatus MPRL 1709	PPY	+++	+++	+++
	VT	++	++	+
B. eggerthii MPRL 1668	PPY	+++	++	+++
	VT	+	+	-
B. variabilis MPRL 1724	PPY	+++	+++	+++
	VT	+	+	-
B. distasonis MPRL 1667	PPY	+++	+	+
	VT	-	-	-

Where +++ = good growth to + = poor growth, - = no growth, VT=Van Tassell & Wilkins medium and PPY= Proteose-peptone yeast extract broth.

3.4 PREPARATION OF LPS LIBRARY

A library of LPS from 8 *Bacteroides* species and one *E. coli* O18K$^-$ strain (grown both aerobically and anaerobically) was prepared from large scale fermenter runs and the LPS extracted by three different methods.

The yield of native LPS obtained for each extraction method is shown in Table 2. The PCP extraction method gave the highest yield of LPS for each organism, with the PW method giving the lowest yield for most organisms. Those organisms that gave a high yield of dry cells were very mucoid and generally gave a poorer yield of LPS. Mucoid organisms were enriched mainly on the 0-20% interface of the Percoll gradients (Fig 5).

The resulting LPSs were analysed by polyacrylamide gels and stained with silver for LPS (Fig 6 A-C) and with Coomassie blue for protein (Fig 7 A, B). Contaminating protein was found in *Bacteroides* LPSs extracted by all three methods, especially those extracted by the PCP and Triton methods (Fig 7). See chemical analysis section 3.6 for levels of protein prior to Proteinase K treatment.

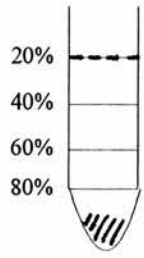
All LPS preparations were then treated with Proteinase K to remove protein contamination after which no Coomassie blue-staining material was observed in PAGE. LPS profiles after silver staining were identical to those before Proteinase K treatment (data not shown). The results in the following sections are from LPSs which had been treated with Proteinase K to remove protein contamination.

Table 2: Details of LPS library

Strain	Growth Medium*	Culture Vol (litres)		Yield from different extraction methods		
				PW	PCP	Triton
B. fragilis NCTC 9343	VT	16	1	6.87	6.87	6.87
			2	41.4	227.5	175.6
			3	0.60	3.31	2.56
B. fragilis NCTC 9343	PPY	15	1	2.34	2.39	2.36
			2	37.4	99.2	47.2
			3	1.60	4.42	2.00
B. fragilis MPRL 1504	PPY	15	1	2.85	2.57	2.50
			2	63.7	64.5	47.6
			3	2.24	2.51	1.91
B. vulgatus MPRL 1985	PPY	15	1	2.42	2.47	2.44
			2	90.7	96.9	53.2
			3	3.74	3.92	2.18
B. thetaiotaomicron MPRL 1720	PPY	15	1	3.23	3.67	3.77
			2	29.8	103.8	83.0
			3	0.92	2.83	2.20
B. caccae MPRL 1555	PPY	15	1	7.40	7.63	7.50
			2	20.3	98.6	20.7
			3	0.27	1.29	0.28
B. uniformis MPRL 1721	PPY	15	1	6.75	5.21	5.60
			2	25.8	213.4	57.7
			3	0.38	4.24	1.03
B. ovatus MPRL 1709	PPY	15	1	3.04	3.00	2.97
			2	54.9	124.0	73.3
			3	0.81	4.14	2.47
E. coli O18K$^-$ MPRL 1275	NB Aerobic	15	1	7.63		
			2	169.8		
			3	2.23		
E. coli O18K$^-$ MPRL 1275	NB Anaerobic	16	1	1.18		
			2	47.5		
			3	4.04		

Where 1= dry weight of cells (g), 2= yield of LPS (mg), 3= % Yield.* VT=Van Tassell & Wilkins' medium, PPY= Proteose-peptone yeast extract broth, NB= nutrient broth, PW= phenol-water, PCP= phenol-chloroform-petroleum .

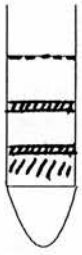
Fig 5: Percoll profiles of fermentation run cultures



B. fragilis
NCTC 9343
VT

B. fragilis
NCTC 9343
PPY

B. fragilis
MPRL 1504
PPY
mucoid



B. vulgatus
MPRL 1985
PPY

B. caccae
MPRl 1555
PPY
very mucoid

B. thetaiotaomicron
MPRL 1720
PPY

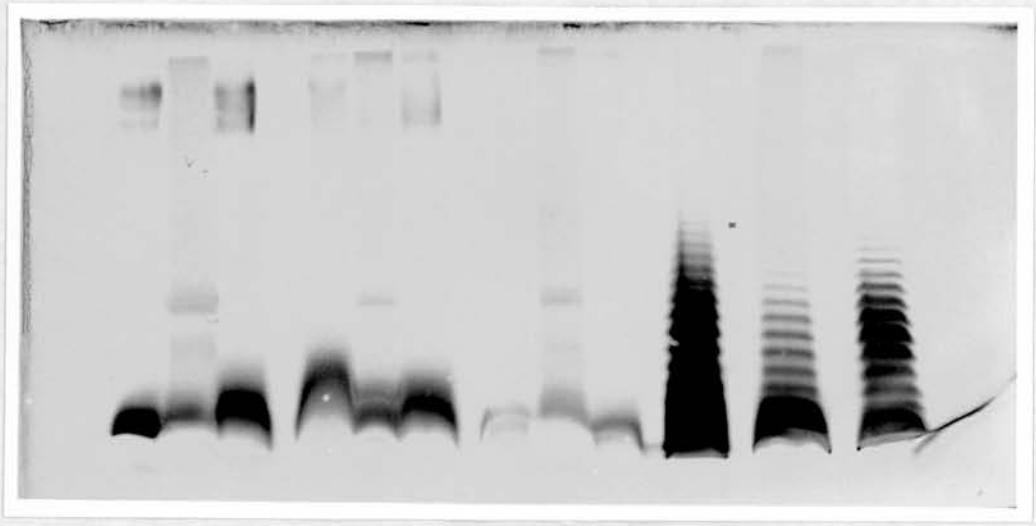
B. uniformis
MPRL 1721
PPY
mucoid

B. ovatus
MPRL 1709
PPY
very mucoid

Where ----- faint band, ▨ thick band and /////// clouding.

Fig 6: Silver stain profile of LPS library

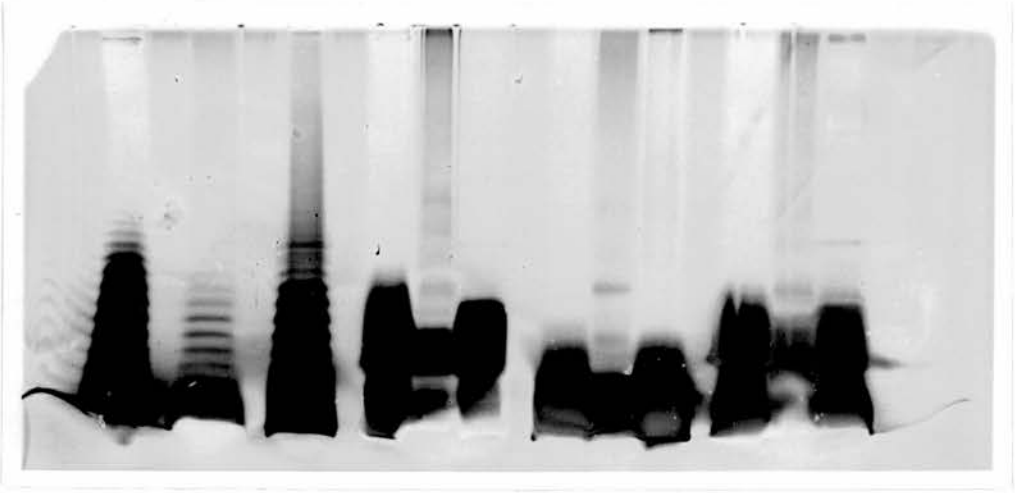
Gel A



Gel A: Track 1= *B. fragilis* NCTC 9343 VT PW, 2= *B. fragilis* NCTC 9343 VT PCP, 3= NCTC 9343 VT Triton, 4= *B. fragilis* NCTC 9343 PPY PW, 5= *B. fragilis* NCTC 9343 PPY PCP, 6= *B. fragilis* NCTC 9343 PPY Triton, 7= *B. fragilis* MPRL 1504 PPY PW, 8= *B. fragilis* MPRL 1504 PPY PCP, 9= *B. fragilis* MPRL 1504 PPY Triton, 10= *B. vulgatus* MPRL 1985 PPY PW, 11= *B. vulgatus* MPRL 1985 PPY PCP, 12= *B. vulgatus* MPRL 1985 PPY Triton.

Where VT= Van Tassell & Wilkins' medium, PPY= Proteose peptone yeast extract broth, NB= nutrient broth, PW= phenol-water, PCP= phenol-chloroform-petroleum, Triton= Triton Mg^{2+}, R = position of rough LPS and X = band present in PCP preparations.

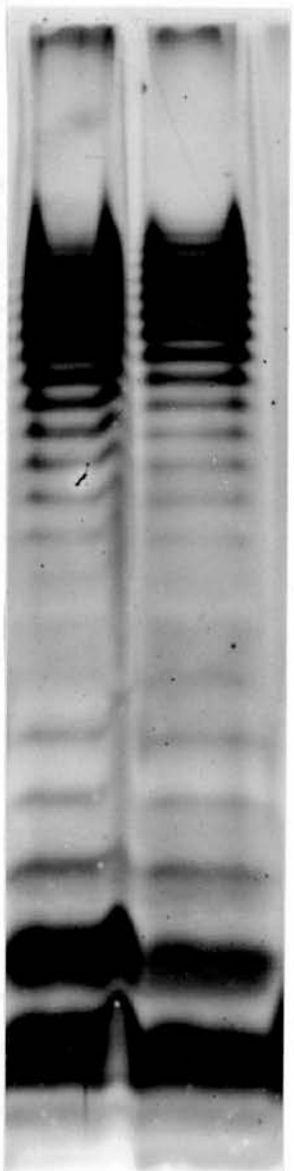
Gel B



1 2 3 4 5 6 7 8 9 10 11 12

Gel B: Track 1= *B. thetaiotaomicron* MPRL 1720 PPY PW, 2= *B. thetaiotaomicron* MPRL 1720 PPY PCP, 3= *B. thetaiotaomicron* MPRL 1720 PPY Triton, 4= *B. caccae* MPRL 1555 PPY PW, 5= *B. caccae* MPRL 1555 PPY PCP, 6= *B. caccae* MPRL 1555 PPY Triton, 7= *B. uniformis* MPRL 1721 PPY PW, 8= *B. uniformis* MPRL 1721 PPY PCP, 9= *B. uniformis* MPRL 1721 PPY Triton, 10= *B. ovatus* MPRL 1709 PPY PW, 11= *B. ovatus* MPRL 1709 PPY PCP, 12= *B. ovatus* MPRL 1709 PPY Triton.

Gel C

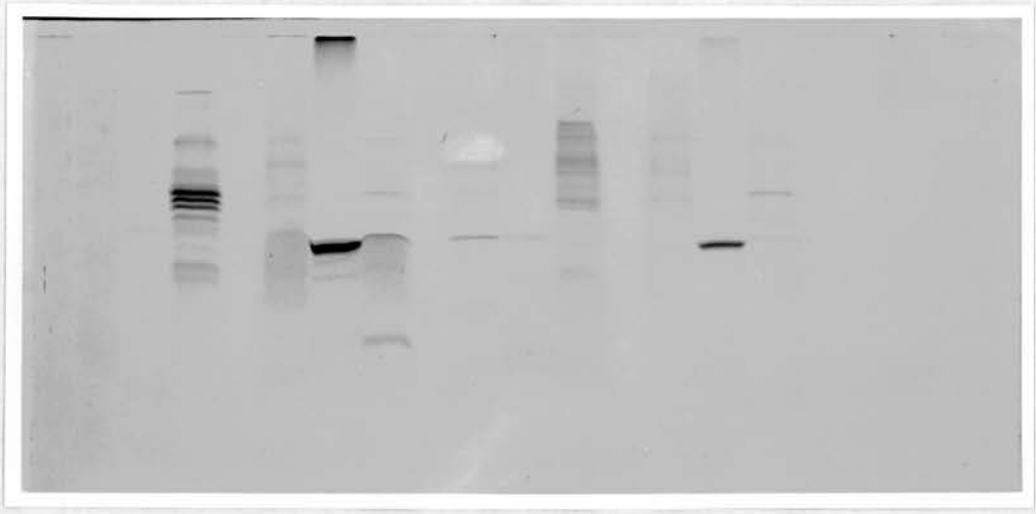


1 2

Gel C: Track 1= *E. coli* O18K$^-$ PW NB grown aerobically, track 2= *E. coli* O18K$^-$ PW NB grown anaerobically.

Fig 7: Coomassie blue profile of LPS library prior to Proteinase K treatment

Gel A

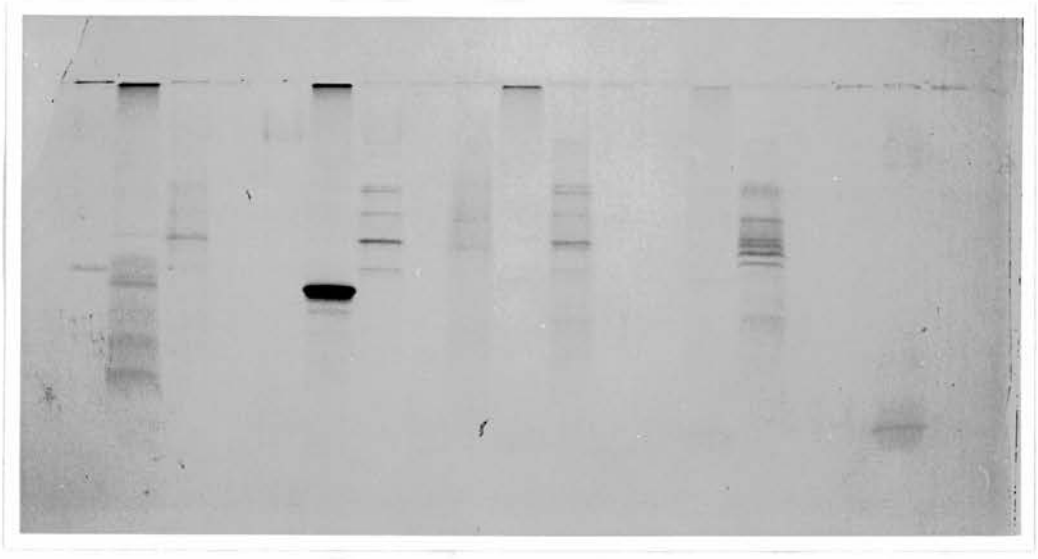


1 2 3 4 5 6 7 8 9 10 11 12

Gel A: Track 1= *B. fragilis* NCTC 9343 VT PW, 2= *B. fragilis* NCTC 9343 VT PCP, 3= NCTC 9343 VT Triton, 4= *B. fragilis* NCTC 9343 PPY PW, 5= *B. fragilis* NCTC 9343 PPY PCP, 6= *B. fragilis* NCTC 9343 PPY Triton, 7= *B. fragilis* MPRL 1504 PPY PW, 8= *B. fragilis* MPRL 1504 PPY PCP, 9= *B. fragilis* MPRL 1504 PPY Triton, 10= *B. vulgatus* MPRL 1985 PPY PW, 11= *B. vulgatus* MPRL 1985 PPY PCP, 12= *B. vulgatus* MPRL 1985 PPY Triton.

Where VT= Van Tassell & Wilkins' medium, PPY= Proteose peptone yeast extract broth, NB= nutrient broth, PW= phenol-water, PCP= phenol-chloroform-petroleum, Triton= Triton Mg^{2+}.

Gel B



1 2 3 4 5 6 7 8 9 10 11 12

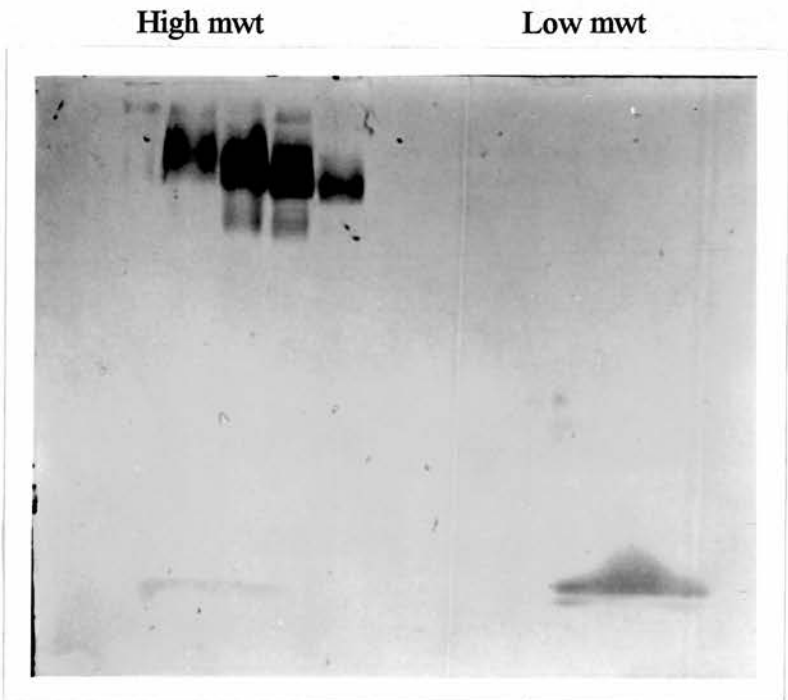
Gel B: Track 1= *B. thetaiotaomicron* MPRL 1720 PPY PW, 2= *B. thetaiotaomicron* MPRL 1720 PPY PCP, 3= *B. thetaiotaomicron* MPRL 1720 PPY Triton, 4= *B. caccae* MPRL 1555 PPY PW, 5= *B. caccae* MPRL 1555 PPY PCP, 6= *B. caccae* MPRL 1555 PPY Triton, 7= *B. uniformis* MPRL 1721 PPY PW, 8= *B. uniformis* MPRL 1721 PPY PCP, 9= *B. uniformis* MPRL 1721 PPY Triton, 10= *B. ovatus* MPRL 1709 PPY PW, 11= *B. ovatus* MPRL 1709 PPY PCP, 12= *B. ovatus* MPRL 1709 PPY Triton.

Fig 6 shows LPS profiles on PAGE for each strain and extraction method after separation and silver staining. A banding pattern typical of smooth LPS was seen for *E. coli* O18K$^-$ (gel C, tracks 1 and 2), *B. vulgatus* MPRL 1985 (gel A, tracks 10, 11 and 12) and *B. thetaiotaomicron* MPRL 1720 (gel B, tracks 1, 2 and 3). Material of high molecular mass was seen in *B. fragilis* species in both the PW and Triton extraction methods. Rough/low molecular mass material is seen for all species by each extraction method, and is "dumbbell shaped" for *B. caccae* and *B. ovatus* (gel B, tracks 4, 5 and 6 and 10, 11 and 12). The PCP extraction method produced a pronounced band (X) present behind the main front band (R) in all preparations. For *B. fragilis* the PCP extraction method did not select for high molecular mass material. Silver stain profiles for sodium salt form LPS were identical to native forms (data not shown). LPS profiles for *E. coli* O18K$^-$ grown aerobically and anaerobically were also identical.

3.5 PREPARATION OF CAPSULAR POLYSACCHARIDE FROM *B. FRAGILIS* NCTC 9343

Capsular polysaccharide was prepared from a 4 litre batch of *B. fragilis* NCTC 9343 which had been grown in PPY medium. Material was fractionated on a Sephacryl (S-300) column and Fig 8 shows fractionated material visualised by silver staining on PAGE. High molecular mass material was seen in fractions 32-40 and low molecular mass material was seen in fractions 50-54. These fractions were pooled, lyophilised and stored at -20°C until future use.

Fig 8: Silver stain profile of fractionated *B. fragilis* NCTC 9343 capsular polysaccharide preparation



Fraction number 30 32 34 36 38 40 42 44 46 48 50 52 54

Void volume= 200 ml

3.6 CHEMICAL ANALYSIS OF LPS LIBRARY

3.6.1 Estimation of Carbohydrate, Phosphorus, KDO and Protein content

Samples of dry extract were resuspended in PF-H_2O to a concentration of 5 mg/ml and assayed for carbohydrate, phosphorus, KDO and protein content. The relative amounts for native LPS samples are shown in Table 3 and for sodium salt LPS samples in Table 4. Before Proteinase K treatment, for native LPSs, contaminating protein was found in PCP extracts from undetectable to > 200 μg/mg dry weight. In all Triton preparations, with the exception of *B. vulgatus* MPRL 1985 in which none was detected, protein contamination was in the range 60-500 μg/mg dry weight and no protein contamination was detectable in the PW extracts. After Proteinase K treatment no protein contamination was detectable in any sample. Batches of sodium salt LPS were prepared after protein decontamination.

Bacteroides species showed a negligible amount or no KDO content before treatment with hydrofluoric acid. Due to sample size KDO measurement was only carried out on selected native *Bacteroides* LPSs (Table 3). Generally the PW extraction method selected for the greatest amount of carbohydrate and KDO material and the PCP extraction method for the least. Phosphorus levels were more uniform among all extraction methods and between native and sodium salt-form LPSs. No difference was observed between LPS extracts from *E. coli* O18K$^-$ grown in different atmospheres.

3.6.2 Estimation of Fatty Acid content

The fatty acids present in native LPSs from *E. coli* O18K$^-$ and selected *Bacteroides* species were analysed by gas liquid chromatography (GLC). Those present and their relative amounts are shown in Table 5. Each *Bacteroides* LPS contained different and varying amounts of fatty acids. In *B. fragilis* NCTC 9343 VT LPS the Triton extraction method selected for greater amounts of fatty acids and the PW method the

Table 3: Estimation of carbohydrate, phosphorus, KDO and protein* content in native LPS samples

LPS Sample	Extraction Method	CHO µg/mg dry wt	P µg/mg dry wt	KDO µg/mg dry wt	Protein* µg/ml dry wt
B. fragilis	PW	415.8	16.4	7.8	-
NCTC 9393	PCP	614.4	33.1	0.8	68.66
VT	Triton	229.2	29.3	3.5	477.61
B. fragilis	PW	172.2	39.2	3.8	-
NCTC 9343	PCP	90.8	33.6	1.4	59.7
PPY	Triton	147.8	49.0	2.1	113.43
B. fragilis	PW	198.2	55.3	6.2	-
MPRL 1504	PCP	120.8	72.4	0.5	-
PPY	Triton	130.0	28.5	3.7	447.76
B. vulgatus	PW	267.6	78.9	2.7	-
MPRL 1985	PCP	67.4	47.4	0.9	-
PPY	Triton	197.2	37.8	2.6	-
B. thetaiotaomicron	PW	363.8	54.2	...	-
MPRL 1720	PCP	43.8	47.4	0.4	179.10
PPY	Triton	262.4	79.6	4.0	226.87
B. caccae	PW	240.2	54.3	...	-
MPRL 1555	PCP	29.6	33.8	...	220.9
PPY	Triton	148.6	24.9	...	226.87
B. uniformis	PW	221.6	102.0	...	-
MPRL 1721	PCP	53.0	38.2	...	-
PPY	Triton	173.4	85.5	...	113.43
B. ovatus	PW	281.0	43.1	5.7	-
MPRL 1709	PCP	110.8	39.6	1.1	-
PPY	Triton	178.0	41.6	2.4	268.66
E. coli O18K$^-$ NB aerobic	PW	254.8	36.5	2.2	-
E. coli O18K$^-$ NB anaerobic	PW	260.0	34.0	2.4	-

Where PW= phenol-water, PCP= phenol-chloroform-petroleum, VT= Van Tassell & Wilkins' medium, PPY= Proteose peptone yeast extract broth, NB= nutrient broth, CHO= carbohydrate, P= phosphorus, ... = not done, - = amount not detectable, *= protein content prior to Proteinase K treatment.

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicates for each experiment.

Table 4: Estimation of carbohydrate and phosphorus content in sodium salt LPS samples

LPS Sample	Extraction Method	CHO µg/mg dry wt	P µg/mg dry wt
B. fragilis	PW	474.8	18.23
NCTC 9393	PCP	19	31.13
VT	Triton	209.4	26.29
B. fragilis	PW	307.2	50.65
NCTC 9343	PCP	30.2	28.87
PPY	Triton	175	29.03
B. fragilis	PW	235.8	34.65
MPRL 1504	PCP	45.6	32.26
PPY	Triton	146.2	34.23
B. vulgatus	PW	375.6	76.13
MPRL 1985	PCP	39.4	40.65
PPY	Triton	281.4	42.90
B. thetaiotaomicron	PW	397.2	51.94
MPRL 1720	PCP	8.0	40.16
PPY	Triton	1.38	43.23
B. caccae	PW	298.0	52.90
MPRL 1555	PCP	28.6	40.32
PPY	Triton	132.0	37.65
B. uniformis	PW	234.4	69.03
MPRL 1721	PCP	42.0	38.71
PPY	Triton	146.2	46.29
B. ovatus	PW	242.0	45.81
MPRL 1709	PCP	85.6	23.84
PPY	Triton	210.6	43.23
E. coli O18K$^-$ NB aerobic	PW	592.4	42.42
E. coli O18K$^-$ NB anaerobic	PW	589.6	42.26

Where PW= phenol-water, PCP= phenol-chloroform-petroleum, VT= Van Tassell & Wilkins' medium, PPY= Proteose peptone yeast extract broth, NB= nutrient broth, CHO= carbohydrate, P= phosphorus, **...** = not done.

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicates for each experiment.

Table 5: Fatty acid content of native PW-LPSs from *E. coli* O18K⁻ and selected *Bacteroides* species

Molar ratio of fatty acid

Strain	12.0	3OH 12.0	13.0	14.0	3OH 14.0	I 15.0	15.0	15.0	16.0	16.1	2OH 16.0	17.0	I 17.0	18.0	18.1
1										1		T			
2	2.1					1	2.1			T					
3	2.0	1.3	1.3			10.6	13.8			2.2					
4	T		T		1	5.2	1			T					
5					T	1									
6		4.6	1.4			13.6	8.3			1.5					1
7	3.7					2.7	1.7	1.8		1	T		T	T	
8	T		T		1					T					

Where 1= *B. fragilis* NCTC 9343 VT PW-LPS
2= *B. fragilis* NCTC 9343 VT PCP-LPS
3= *B. fragilis* NCTC 9343 VT Triton-LPS
4= *B. fragilis* NCTC 9343 PPY PW-LPS
5= *B. caccae* MPRL 1555 PPY PW-LPS
6= *B. thetaiotaomicron* MPRL 1720 PPY PW-LPS
7= *B. vulgatus* MPRL 1985 PPY PW-LPS
8= *E. coli* O18K⁻ grown aerobically PW-LPS
T= Trace amount
I = iso
OH = hydroxy

See Table 3 for other abbreviations.

least. The *Bacteroides* LPSs predominately contained larger amounts of Iso-pentadecanoic, pentadecanoic and dodecanoic fatty acids. Small trace amounts of 3-hydroxytetradecanoic acid, the predominant fatty acid in *E. coli* O18K$^-$ LPS was found in *B. fragilis* NCTC 9343 PPY PW-LPS and *B. caccae* MPRL 1555 PW-LPS.

3.7 CHEMICAL ANALYSIS OF CAPSULE PREPARATION FROM *B. FRAGILIS* NCTC 9343

Samples of dry high molecular mass and low molecular mass material were resuspended to 5 mg/ml and assayed for carbohydrate, phosphorus, KDO and protein content. The relative amounts of each are shown in Table 6. No protein contamination was found in either the high or low molecular mass material. The low molecular mass material contained more carbohydrate and phosphorus; and only the low molecular mass material contained detectable KDO.

Table 6: Estimation of carbohydrate, phosphorus, KDO and protein in capsular preparation from *B. fragilis* NCTC 9343

Sample	CHO μg/mg	P μg/mg	KDO μg/mg	Protein μg/mg
High molecular mass material	101.4	2.07	-	-
Low molecular mass material	146.34	36.12	6.88	-

Where - = non-detectable. See Table 3 for other abbreviations.

3.8 LPS INDUCED MOUSE LETHALITY (GALACTOSAMINE MODEL)

E. coli O18K$^-$ LPS was lethal for all mice at 50 ng/mouse (Table 7) whereas *B. fragilis* NCTC 9343 VT PW-LPS and *B. vulgatus* MPRL 1985 PW-LPS were only lethal to mice at 20 μg/mouse. No difference in lethality was seen for native and sodium salt-form *B. fragilis* NCTC 9343 VT PW-LPS. Mice were not killed by the maximum dose of *B. fragilis* LPS extracted by the Triton or PCP method, nor by *B. uniformis* and *B. ovatus* LPS extracted by the PW method.

Table 7: LPS induced mouse lethality (D-gal N Model)

LPS Sample	Survivors after dose of LPS of				
	5 ng	50 ng	5 μg	10 μg	20 μg
B. fragilis NCTC 9343 VT PW	...	...	3/3	1/3	0/3
B. fragilis NCTC 9343 PPY PW	...	...	3/3	2/3	1/3
B. fragilis MPRL 1504 PW	...	...	3/3	3/3	2/3
B. vulgatus MPRL 1985 PW	...	...	3/3	3/3	0/3
B. thetaiotaomicron MPRL 1720 PW	...	...	3/3	3/3	1/3
B. caccae MPRL 1555 PW	...	...	3/3	3/3	1/3
B. uniformis MPRL 1721 PW	...	...	3/3	3/3	3/3
B. ovatus MPRL 1709 PW	...	...	3/3	3/3	3/3
B. fragilis NCTC 9343 PPY PCP	...	...	3/3	3/3	3/3
B. fragilis NCTC 9343 PPY Triton	...	...	3/3	3/3	3/3
**B. fragilis* NCTC 9343 PPY PW	...	...	3/3	2/3	0/3
**B. fragilis* NCTC 9343 PPY PCP	...	...	3/3	3/3	3/3
**B. fragilis* NCTC 9343 PPY Triton	...	...	3/3	3/3	3/3
E. coli O18K$^-$ aerobic PW	3/3	0/3	0/3	...	...
E. coli O18K$^-$ anaerobic PW	3/3	0/3	0/3	...	...

All LPS are native samples unless indicated. *sodium salt-form LPS, see Table 3 for other abbreviations.

3.9 REACTIVITY OF LPS SAMPLES IN LAL ASSAY

3.9.1 Reactivity of Native *Bacteroides* LPSs Before and After Protein Decontamination

Endotoxic activities in the LAL assay of native *Bacteroides* LPSs before and after protein decontamination are illustrated in Table 8. In the vast majority of cases the activity of LPS samples increased after protein decontamination. On a weight for weight basis, the greatest activity was seen in the PW extracts and the least activity in PCP extracts. The differences in reactivity between protein-decontaminated *Bacteroides* LPSs and *E. coli* O18K$^-$ LPS are discussed more fully in section 3.9.2 below.

3.9.2 Reactivity of Native vs Sodium Salt-Form LPS Extracted by Three Different Methods

Endotoxic activities in the LAL assay of native and sodium salt form LPS are illustrated in Table 9 (i), (ii) and (iii) for each extraction method. On a weight for weight basis, the greatest activity was seen in the PW extracts and the least activity seen in the PCP extracts. All native PW-LPSs were more active than sodium salt PW-LPSs. Approximately 70% of native PCP-LPSs were more active than sodium salt PCP-LPSs. No significant difference between native and sodium salt-form Triton LPSs was seen. Aerobically grown *E. coli* O18K$^-$ LPS was more active than anaerobically grown *E. coli* O18K$^-$ LPS by a factor of two. Comparing PW LPSs, *B. fragilis* MPRL 1504 PW-LPS was more active than *E. coli* O18K$^-$ LPS by a factor of 30, *B. fragilis* NCTC 9343 VT PW-LPS by a factor of 10, *B. fragilis* NCTC 9343 PPY PW-LPS by a factor of five and *B. caccae* MPRL 1555 PW-LPS by a factor of four. All other *Bacteroides* species were less active than *E. coli* O18K$^-$. A difference in activity for *B. fragilis* NCTC 9343 LPS grown in different media was also seen.

Table 8: Endotoxic activities in LAL (IU/ml) of native *Bacteroides* LPSs (5 ng/ml) before and after protein decontamination

			LAL activity (IU/ml)	
Sample	Growth Medium	Extraction Method	Before protein decontamination	After protein decontamination
B. fragilis NCTC 9343	VT	PW	426.3	617.4
		PCP	0	0.449
		Triton	236.2	480.4
B. fragilis NCTC 9343	PPY	PW	192.72	227.8
		PCP	0.012	0.608
		Triton	58.33	71.75
B. fragilis MPRL 1504	PPY	PW	729.56	1863
		PCP	0	0.90
		Triton	510.11	704
B. vulgatus MPRL 1985	PPY	PW	8.44	15.59
		PCP	0	0.34
		Triton	9.96	11.05
B. thetaiotaomicron MPRL 1720	PPY	PW	3.18	4.11
		PCP	0	0.80
		Triton	15.67	17.38
B. caccae MPRL 1555	PPY	PW	181.04	234.9
		PCP	0	0.08
		Triton	51.26	50.64
B. uniformis MPRL 1721	PPY	PW	10.40	13.72
		PCP	2.63	20.74
		Triton	41.11	42.33
B. ovatus MPRL 1709	PPY	PW	3.85	4.27
		PCP	0	0.56
		Triton	39.90	46.33

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for all assays. See Table 3 for other abbreviations.

Table 9: Endotoxic activities in LAL (IU/ml) of sodium salt and native LPS (5 ng/ml) samples

(i) PW Extracts.

	LAL activity	(IU/ml)
LPS Sample	Sodium salt form	Native form
B. fragilis NCTC 9343 VT	75.4	617.4
B. fragilis NCTC 9343 PPY	48.7	227.8
B. fragilis MPRL 1504	136.2	1863.0
B. vulgatus MPRL 1985	3.3	15.6
B. thetaiotaomicron MPRL 1720	1.0	4.1
B. caccae MPRL 1555	67.4	234.9
B. uniformis MPRL 1721	0.9	13.7
B. ovatus MPRL 1709	12.5	4.3
E. coli O18K$^-$ aerobically grown PW	6.8	62.2
E. coli O18K$^-$ anaerobically grown PW	2.2	31.7

(ii) PCP Extracts

	LAL activity	(IU/ml)
LPS Sample	Sodium salt form	Native form
B. fragilis NCTC 9343 VT	1.1	0.5
B. fragilis NCTC 9343 PPY	4.9	0.6
B. fragilis MPRL 1504	0.8	0.9
B. vulgatus MPRL 1985	4.2	0.4
B. thetaiotaomicron MPRL 1720	0.2	0.8
B. caccae MPRL 1555	0.2	0.1
B. uniformis MPRL 1721	7.9	20.7
B. ovatus MPRL 1709	0.1	0.6
E. coli O18K$^-$ aerobically grown PW	10.4	45.4
E. coli O18K$^-$ anaerobically grown PW	6.0	23.9

(iii) Triton Extracts

	LAL activity	(IU/ml)
LPS Sample	Sodium salt form	Native form
B. fragilis NCTC 9343 VT	226.2	480.4
B. fragilis NCTC 9343 PPY	455.7	71.7
B. fragilis MPRL 1504	1020.0	704.0
B. vulgatus MPRL 1985	34.6	11.1
B. thetaiotaomicron MPRL 1720	63.5	17.4
B. caccae MPRL 1555	6.0	50.6
B. uniformis MPRL 1721	22.2	42.3
B. ovatus MPRL 1709	124.4	46.3
E. coli O18K$^-$ aerobically grown PW	9.2	83.6
E. coli O18K$^-$ anaerobically grown PW	8.8	42.3

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for all assays. The above results refer to LPSs which had been protein decontaminated. See Table 3 for other abbreviations.

3.10 LPS-INDUCED TNF SECRETION

3.10.1 Inter- and Intra-Assay Variation

A degree of inter-assay variation in the TNF was unavoidable due to variations between individual blood donors (this ranged from 5% to 45%, data not shown) and between cell cultures produced on different occasions. Where the results have not been analysed statistically, the results presented are the mean of two experiments performed with the same cell preparations on the same day. All assays were repeated on at least two occasions with cell supernates derived from different stimulation experiments. All results presented depict trends which have been confirmed on at least two occasions. Due to the heterogeneity in molecular mass of the *E. coli* LPS (ladder pattern) and the complexity of the appearance on SDS-PAGE (Fig 6) and unknown chemical structure and molecular composition of the *Bacteroides* LPS complex, the following results are compared on a weight for weight basis, rather than a molar basis.

3.10.2 TNF Production by Protein-Contaminated and -Decontaminated LPSs

The effect of protein contamination of native *Bacteroides* PW-LPSs on TNF production was examined in human mononuclear leukocytes (MNL) (Table 10). In all cases the amount of TNF produced increased after protein decontamination. Trends between species remained the same. *B. fragilis* NCTC 9343 VT PW-LPS induced the most TNF and *B. vulgatus* MPRL 1985 the least. The differences between the amount of TNF induced by different LPSs is discussed more fully in section 3.10.5. The following results in section 3.10 are obtained from protein decontaminated samples only.

3.10.3 Effect of Different Sera and Different Experimental Volumes on TNF Production

The effect of different sera in the culture medium on TNF production was examined in human MNL after stimulation with various LPSs (Table 11). The amount of TNF produced by all LPSs tested did not alter when FCS was replaced with human serum. The amount of TNF produced by all *Bacteroides* LPSs was not affected by the lack of serum in the culture medium, however the amount of TNF produced by *E. coli* O18K$^-$ LPS was approximately halved when serum was absent from the culture medium.

The effect of different experimental volumes on TNF production was examined in human MNL after stimulation with various LPSs (Table 12). The largest amount of TNF was produced when the experiment was conducted in the wells of 96 well microtitre plates which had an experimental volume size of 200 µl. The amount of TNF produced decreased as the experimental volume size increased. All experiments in results conducted in 96 well plates unless otherwise indicated.

3.10.4 Effect of Amount of LPS on TNF Production

The effect of varying ng amounts of native PW-LPS on TNF production was examined for *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 (Fig 9). In all cell populations tested, more TNF was produced as LPS levels increased. For human MNL (Fig 9 a) *E. coli* O18K$^-$ LPS produced more TNF than *B. fragilis* NCTC 9343 LPS for all amounts tested. No difference in TNF production by *B. fragilis* NCTC 9343 LPS was seen for THP-1 cells with and without enhancement for CD14 (Fig 9 b). The level of TNF production by *E. coli* O18K$^-$ LPS in THP-1 cells without enhancement for CD14 was comparable to that produced by *B. fragilis* NCTC 9343 LPS. However, the level of TNF production by *E. coli* O18K$^-$ LPS was greatly increased in THP-1 cells with enhancement for CD14 compared to *B. fragilis* NCTC 9343. No major

Table 10: Measured TNF concentration (IU/ml) from human MNL after stimulation for 4 h with native PW-LPS (50 ng/ml) from *Bacteroides* species before and after protein decontamination

LPS	TNF concentration IU/ml + protein contamination	TNF concentration IU/ml - protein contamination
B. fragilis NCTC 9343 VT	19.15	23.16
B. fragilis NCTC 9343	17.7	18.82
B. fragilis MPRL 1504	3.99	4.93
B. vulgatus MPRL 1985	2.41	2.58
B. thetaiotaomicron MPRL 1720	5.88	6.4
B. caccae MPRL 1555	11.27	13.89
B. uniformis MPRL 1721	9.832	10.1
B. ovatus MPRL 1709	6.21	8.66

Table 11: Measured TNF concentration (IU/ml) from human MNL after stimulation for 4 h with various native PW-LPSs (50 ng/ml) from *Bacteroides* species and *E. coli* O18K$^-$ in the presence and absence of FCS and human serum

LPS	TNF concentration (IU/ml)		
	- serum	+ FCS	+ human serum
B. fragilis NCTC 9343	48.97	42.66	45.92
B. vulgatus MPRL 1985	9.26	10.18	11.54
B. thetaiotaomicron MPRL 1720	7.28	9.23	8.77
B. caccae MPRL 1555	29.04	27.78	26.10
E. coli O18K$^-$	48.97	101.01	104.19

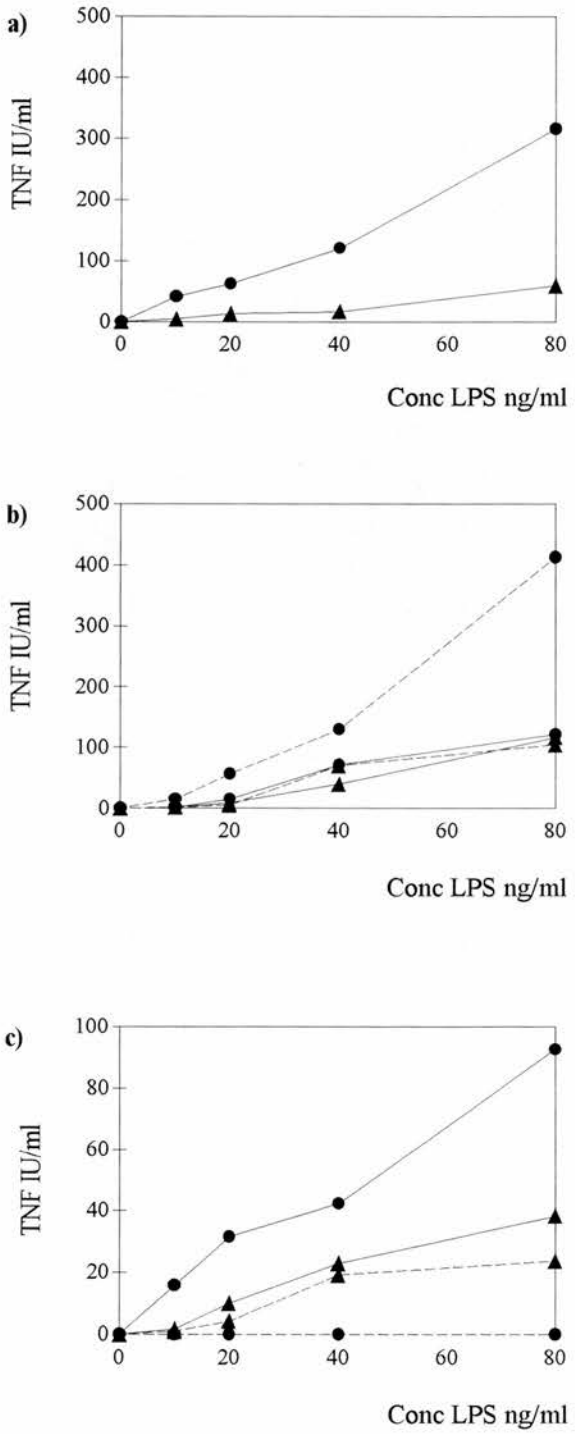
Each value in Tables 10 and 11 are the mean of duplicate experiments performed with the same cell preparations.

Table 12: Measured TNF concentration (IU/ml) from human MNL after stimulation for 4 h with various native PW-LPSs (50 ng/ml) from *Bacteroides* species and *E. coli* O18K⁻ in different experimental volumes*

	TNF	concentration	(IU/ml)
LPS	96 well plate (200 µl)*	24 well plate (1 ml)*	tube (2 ml)*
B. fragilis NCTC 9343	27.88	15.04	14.87
B. vulgatus MPRL 1985	4.98	2.86	0
B. thetaiotaomicron MPRL 1720	4.55	2.83	1.32
B. caccae MPRL 1555	14.74	8.78	7.87
E. coli O18K⁻	103.04	83.27	78.75

Each value is the mean of duplicate experiments performed with the same cell preparation.

Fig 9: TNF production (IU/ml) after 4 h by: a) human MNL, b) THP-1 cells with (---) and without (__) enhancement for CD14, and c) peritoneal macrophages from C3H/HeJ (---) and C3H/HeN (__) mice after stimulation with native PW-LPS from *E. coli* O18K⁻ (●) and *B. fragilis* NCTC 9343 (▲). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.



difference in TNF production by *B. fragilis* NCTC 9343 LPS was seen between peritoneal macrophages from C3H/HeJ (LPS non-responder) and C3H/HeN (LPS responder) mice (Fig 9 c), but consistently there was slightly more TNF produced by the C3H/HeN cells. Note the different scales of the y axes. There was no TNF production observed in C3H/HeJ peritoneal macrophages stimulated by *E. coli* O18K$^-$ LPS. However, in peritoneal macrophages from C3H/HeN mice, the level of TNF production was greatly increased after stimulation by *E. coli* O18K$^-$ LPS. The amount of TNF produced by peritoneal macrophages after stimulation by all LPSs tested was considerably lower than that produced by the other cell populations.

3.10.5 TNF Production by *Bacteroides* LPSs Extracted by Three Different Methods

Based on the data from Fig 9, a LPS concentration of 50 ng/ml was selected to compare a larger panel of LPSs from other *Bacteroides* species and enterobacteria (Table 13). This value was chosen as it was in the middle of the linear part of both the dose response curve and the standard curve of the assay system employed. This would not show the maximum induction capacity of the LPSs but was a compromise for comparing a large number of LPS samples. It is recognised that this level of LPS is high and may not reflect the situation *in vivo* in the bloodstream but may mimic the situation in abscesses where the local concentration of LPS is presumed to be higher. *E. coli* O18K$^-$ stimulated the most TNF production with no difference in TNF production between *E. coli* O18K$^-$ LPS grown aerobically or anaerobically. On a weight for weight basis, PW-LPS stimulated the greatest TNF production. Comparing PW-LPS, *Bacteroides* LPS induced TNF on a magnitude much less than *E. coli* O18K$^-$, with the most active (*B. fragilis* NCTC 9343 VT) being four times less active than *E. coli* O18K$^-$ and the least active (*B. vulgatus* MPRL 1985) being 37 times less active than *E. coli* O18K$^-$. TNF production of other enterobacterial LPSs tested was of the same level or less than *E. coli* O18K$^-$, all being more active than *Bacteroides*

Table 13: Measured TNF concentration (IU/ml) from human MNL after 4 h stimulation with native and sodium salt-form LPS (50 ng/ml) from *E. coli* O18K⁻, various *Enterobacteriaceae* and *Bacteroides* species extracted by three different methods.

		TNF produced (IU/ml)	
LPS Sample	Extraction Method	Native LPS	Sodium Salt LPS
B. fragilis NCTC 9343 VT	PW	23.16	11.92
	PCP	0.30	0.71
	Triton	2.99	1.07
B. fragilis NCTC 9343 PPY	PW	18.81	4.60
	PCP	2.53	1.78
	Triton	0.51	0.31
B. fragilis MPRL 1504	PW	4.93	2.51
	PCP	0.42	0.25
	Triton	0.99	0.47
B. vulgatus MPRL 1985	PW	2.58	0.72
	PCP	0.15	0.51
	Triton	0.39	0.31
B. thetaiotaomicron MPRL 1720	PW	6.40	6.47
	PCP	0.03	0.43
	Triton	0.17	0.49
B. caccae MPRL 1555	PW	13.89	15.25
	PCP	0.22	0.74
	Triton	2.48	2.44
B. uniformis MPRL 1721	PW	10.10	35.34
	PCP	4.81	4.81
	Triton	10.87	5.09
B. ovatus MPRL 1709	PW	8.66	13.89
	PCP	1.44	5.37
	Triton	1.25	2.32
E. coli O18 K⁻ aerobically grown	PW	94.61	43.21
E. coli O18K⁻ anaerobically grown	PW	99.00	43.21
E. coli O6	PW	59.00	...
E. coli O12	PW	58.20	...
E. coli O15	PW	68.96	...
K. pneumoniae M10B	PW	48.21	...
E. coli J5	PCP	36.63	...
E. coli K12	PCP	49.90	...
S. minnesota Ra	PCP	31.68	...
S. minnesota Rb	PCP	96.70	...
S. minnesota Rc	PCP	49.90	...
S. minnesota Rd	PCP	78.45	...
S. minnesota Re	PCP	50.48	...

All assays were carried out on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for each assay. See Table 3 for other abbreviations.

LPS. Sodium salt-form *E. coli* O18K⁻ LPS had half the activity of native form *E. coli* O18K⁻ LPS. Generally for B*acteroides* LPS there is no clear distinction in activity between native and sodium salt-form LPS.

3.10.6 Time course of TNF Production

Levels of TNF production by human MNL were measured at hourly intervals after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ and *B. fragilis* NCTC 9343 (Fig 10 a). The response produced by the *E. coli* LPS showed a complex periodic rise and fall with major peaks at between three and five hours and eight hours. The *B. fragilis* NCTC 9343 LPS showed one major peak between three and six hours with a minor rise at eight hours. This type of response for *E. coli* O18K⁻ LPS has been seen by other workers in other laboratories (Gardiner *et al* 1991 and Dr Isla Halliday, Queen's University of Belfast, personal communication) and a second peak at eight hours has been a consistent finding and has been reproduced on numerous occasions in our laboratories by several different workers. To confirm the periodicity of the TNF response to *E. coli* O18K⁻ LPS the experiment was repeated on four occasions with MNLs from different donors, sampling at two-hourly intervals until eight hours and then at 20 hours (Fig 10 b, c). All the mean TNF values in IU/ml from duplicate estimations were normalised to the value for *E. coli* O18K⁻ LPS at 4 h which was given the arbitrary value of 100%. Statistical analysis by the students t test for paired means showed that the differences between peaks and troughs for the response to *E. coli* O18K⁻ LPS were highly significant: between 2 h and 4 h $p=0.0027$, between 4 h and 6 h $p=0.0032$ and between 6 h and 8 h $p=0.0312$ where $p \leq 0.05$ is significant.

TNF induction by LPSs from other *Bacteroides* species and *E. coli* O18K⁻ were also followed by sampling at two hourly intervals in other cell populations (Fig 11 a-d).

Fig 10: Time course of TNF production after stimulation of human MNL with LPS (50 ng/ml) from *E. coli* O18K⁻ (●) or *B. fragilis* NCTC 9343 (▲). a) Samples taken hourly over 24 h from a single experiment where TNF values in IU/ml are the means of duplicate TNF estimations (vertical bars indicate the difference between duplicate estimations). b) and c) Results from four separate experiments performed on different occasions with different cell preparations. Each point is the mean of two TNF estimations and all values have been normalised relative to the 4 h value for *E. coli* LPS which has been defined as 100%.

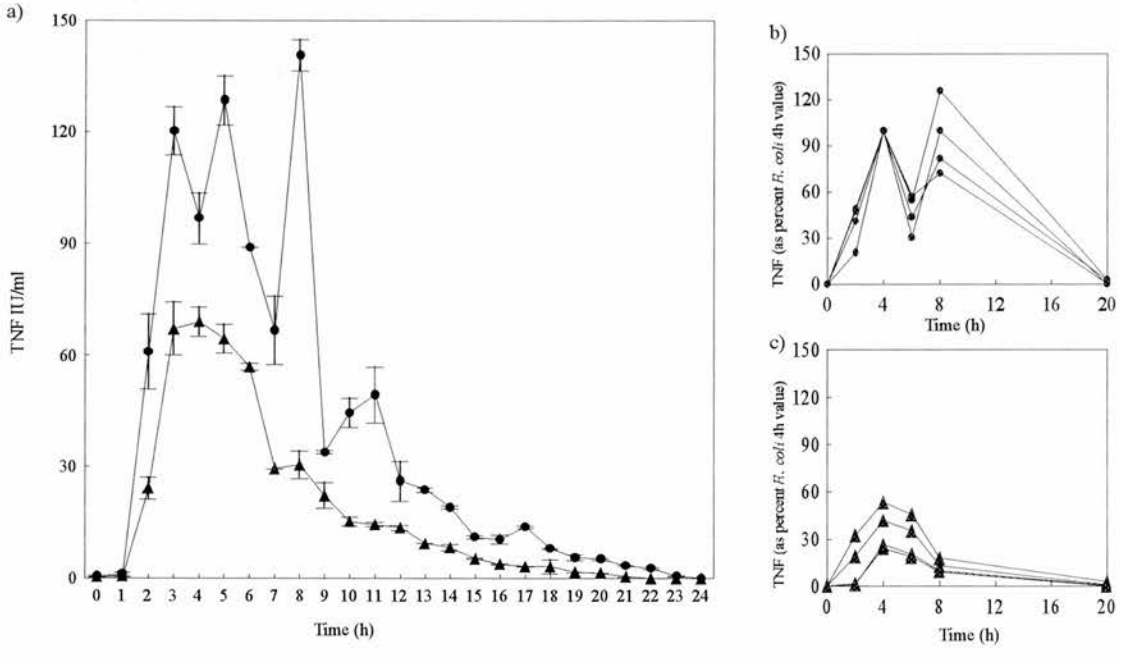
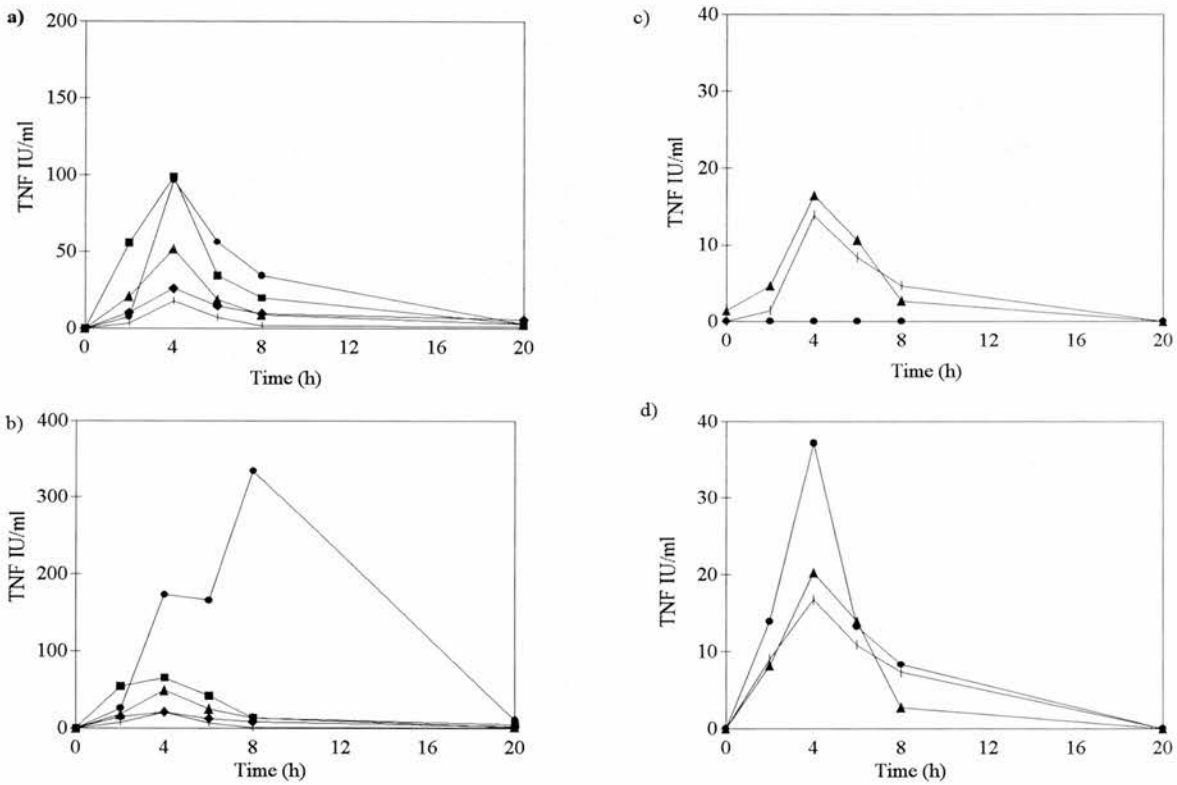


Fig 11: Time course of TNF production (IU/ml) by: a) THP-1 cells without enhancement for CD14, b) THP-1 cells with enhancement for CD14, c) peritoneal macrophages from C3H/HeJ mice and d) peritoneal macrophages from C3H/HeN mice after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (●), *B. fragilis* NCTC 9343 (▲), *B. caccae* MPRL 1555 (◆), *B. uniformis* MPRL 1721 (■), and *B. vulgatus* MPRL 1985 (|). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.

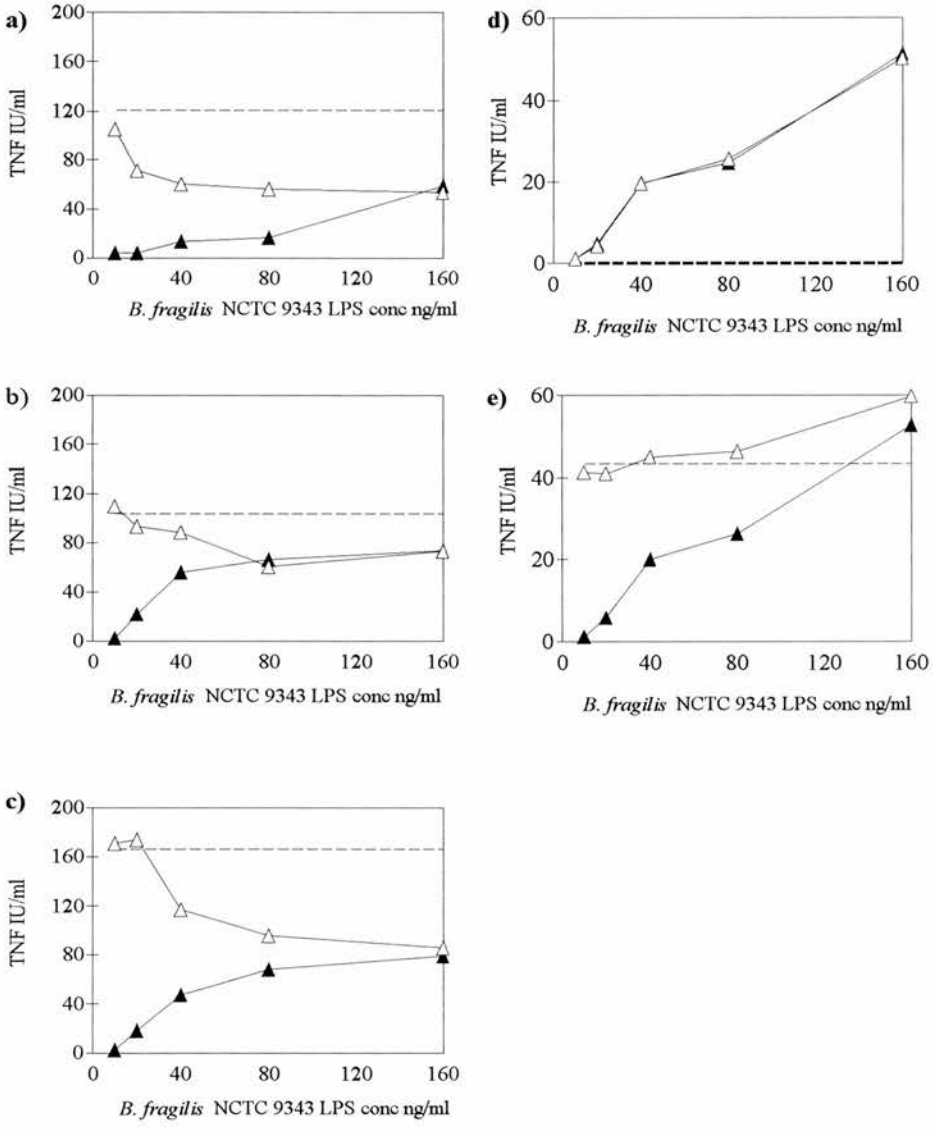


For THP-1 cells without enhancement for CD14, only one peak of TNF was stimulated at 4 h for all LPSs tested. LPSs from *E. coli* O18K⁻ and *B. uniformis* MPRL 1721 were the most active and LPS from *B. vulgatus* being the least active (Fig 11 a). In THP-1 cells with enhancement for CD14 a peak of TNF production was seen at 4 h for all LPSs tested. However, a second peak of TNF production, larger than the first was seen at 8 h for *E. coli* O18K⁻ only. LPS from *E. coli* O18K⁻ was approximately three- to five-fold more active than the most active *Bacteroides* LPS (Fig 11 b). In peritoneal macrophages from both C3H/HeJ (LPS non-responder) and C3H/HeN (LPS responder) mice a peak of TNF production was seen at 4 h for the LPSs tested (Fig 11 c, d), with the exception of *E. coli* O18K⁻ in C3H/HeJ peritoneal macrophages where no TNF was produced. In peritoneal macrophages from C3H/HeN mice, *E. coli* O18K⁻ LPS was only 2-fold more active than *B. fragilis* NCTC 9343 LPS. The difference in activity between *B. fragilis* NCTC 9343 and *B. vulgatus* MPRL 1985 LPS was not as marked as previously seen. In most cell populations the amount of TNF production fell to zero more quickly for *Bacteroides* LPS than *E. coli* O18K⁻ LPS. *B. fragilis* NCTC 9343 LPS which had been extracted by the PCP and Triton methods also produced a single peak of TNF at 4 h from human MNL but on a magnitude much less than that seen for the PW-LPS (data not shown).

3.10.7 Effect of TNF Production by *E. coli* O18K⁻ LPS With *B. fragilis* NCTC 9343 LPS Present in Excess

When LPS from *B. fragilis* NCTC 9343 was in excess of a constant amount of *E. coli* O18K⁻ LPS (40 ng/ml) in human MNL and THP-1 cells with and without enhancement for CD14, TNF production was comparable to that of *B. fragilis* NCTC 9343 LPS alone and not that of *E. coli* O18K⁻ alone (Fig 12 a, b, c). There appeared to be a lowering and masking effect of TNF production from *E. coli* O18K⁻ LPS when LPS from *B. fragilis* NCTC 9343 was present in excess. This trend was also

Fig 12: TNF production (IU/ml) after 4 h by a) human MNL b) THP-1 cells without enhancement for CD14, c) THP-1 cells with enhancement for CD14, d) peritoneal macrophages from C3H/HeJ mice and e) peritoneal macrophages from C3H/HeN mice after stimulation with varying amounts of LPS from *B. fragilis* NCTC 9343 (▲), varying amounts of *B. fragilis* NCTC 9343 LPS together with a constant amount of *E. coli* O18K⁻ (40 ng/ml) (Δ). Maximum amount of TNF produced by *E. coli* O18K⁻ alone shown by (---). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.

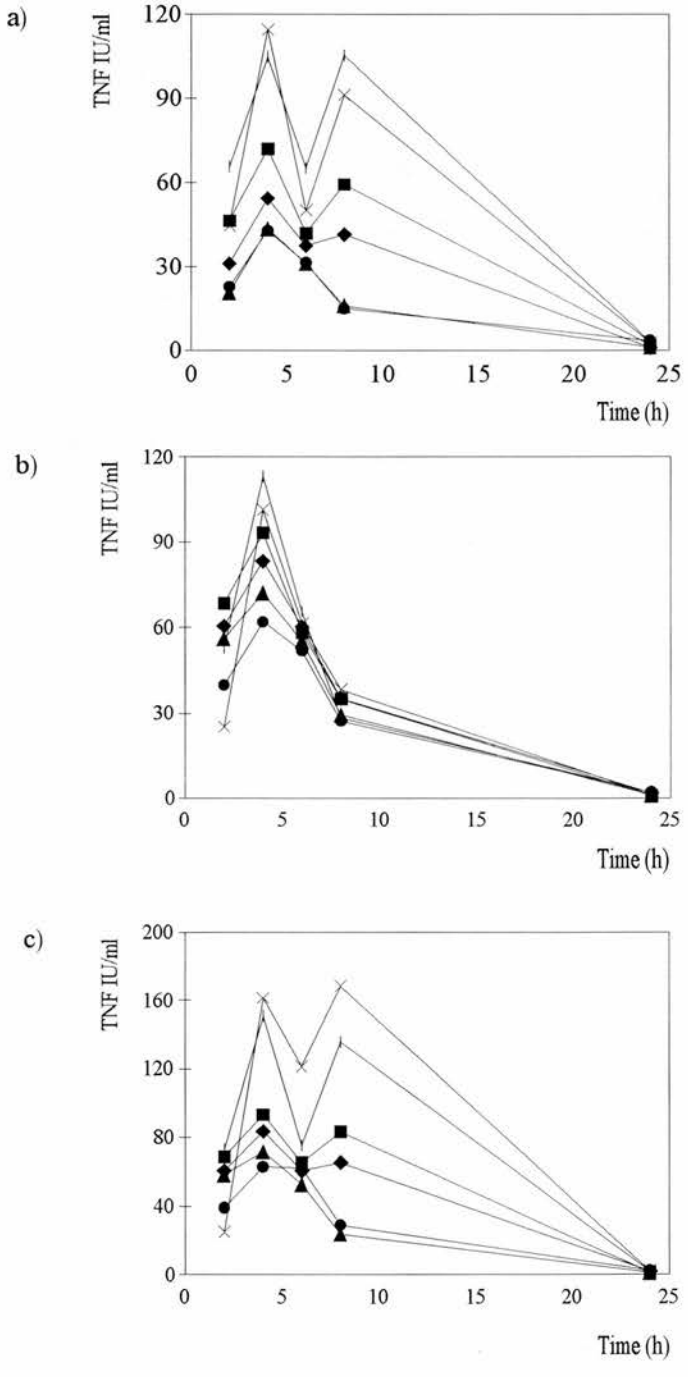


seen for *B. vulgatus* MPRL 1985 and *B. caccae* MPRL 1555 PW-LPS (data not shown). A masking effect was still observed for *B. fragilis* NCTC 9343 LPS which had been extracted by the PCP and Triton methods. In this case TNF production was approximately half the normal level of that induced by *E. coli* O18K⁻ LPS (not comparable to *B. fragilis* NCTC 9343 LPS alone) when *B. fragilis* NCTC 9343 LPS was in excess. For peritoneal macrophages from C3H/HeJ mice TNF production for *B. fragilis* NCTC 9343 LPS alone and *B. fragilis* NCTC 9343 LPS together with *E. coli* O18K⁻ LPS were identical: *E. coli* O18K⁻ LPS alone does not cause TNF stimulation (Fig 12 d). The trend seen in the other cell populations was not clear for peritoneal macrophages from C3H/HeN mice as the amount of TNF produced by *E. coli* O18K⁻ LPS alone is lower than that of the highest concentration of *B. fragilis* NCTC 9343 LPS (Fig 12 e).

3.10.8 Time course of TNF Production by *E. coli* O18K⁻ LPS With *B. fragilis* NCTC 9343 LPS Present in Excess

When LPS from *B. fragilis* NCTC 9343 was present in excess of a constant amount of *E. coli* O18K⁻ LPS (40 ng/ml) in human MNL and THP-1 cells with enhancement for CD14, TNF production was greatly reduced and produced only one peak of TNF at 4 h (Fig 13 a, c). The second peak of TNF production at 8 h returned as the amount of *B. fragilis* NCTC 9343 LPS present with *E. coli* O18K⁻ LPS decreased until it is was no longer present in excess. There was no effect on the kinetics of TNF production when LPS from *B. fragilis* NCTC 9343 was present in excess of a constant amount of *E. coli* O18K⁻ LPS (40 ng/ml) in THP-1 cells without enhancement for CD14 (Fig 13 b). In this cell population TNF production was greatly reduced when *B. fragilis* NCTC 9343 LPS was present in excess and a peak of TNF at 4 h was observed in all cases.

Fig 13: Time course of TNF production (IU/ml) by a) human MNL, b) THP-1 cells without enhancement for CD14 and c) THP-1 cells with enhancement for CD14 after stimulation with 10 ng/ml (l), 20 ng/ml (■), 40 ng/ml (◆), 80 ng/ml (●) and 160 ng/ml (▲) of LPS from *B. fragilis* NCTC 9343 together with a constant amount of *E. coli* O18K⁻ (40 ng/ml). TNF produced by *E. coli* O18K⁻ alone shown by (X). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.



3.10.9 TNF Production by *E. coli* O18K⁻ LPS With *B. fragilis* NCTC 9343 LPS Present in Excess at Various Time Intervals

The effect of delaying the addition of an excess amount of *B. fragilis* NCTC 9343 LPS (160 ng/ml) to *E. coli* O18K⁻ LPS (40 ng/ml) in stimulating TNF production after 4 h from human MNL and THP-1 cell populations was examined (Table 14). TNF production was most greatly reduced in all cell populations when an excess of *B. fragilis* NCTC 9343 LPS was added at the same time as *E. coli* O18K⁻ LPS. A masking effect was still observed in all cell populations but slightly reduced when *B. fragilis* NCTC 9343 LPS was added 2-3 h after the addition of *E. coli* O18K⁻ LPS to cell populations.

3.10.10 Inhibition of TNF Production by an Anti-CD14 mAb

The effect of a CD14 mAb in inhibiting TNF production was examined. In all cell populations tested, the presence of a CD14 mAb did not affect the production of TNF stimulated by *B. fragilis* NCTC 9343 LPS (Fig 14). However, the production of TNF stimulated by *E. coli* O18K⁻ was inhibited as the concentration of CD14 mAb increased. This inhibition of TNF production was more marked in human MNL and THP-1 cells with enhancement for CD14 (Fig 14 a, c). A small amount of background TNF was produced by the CD14 mAb alone.

3.10.11 Time course of TNF Production After Inhibition by an Anti-CD14 mAb

In the presence of high levels of anti-CD14 mAb, the 8 h peak of TNF production after stimulation with *E. coli* O18K⁻ LPS seen in human MNL and THP-1 cells with enhancement for CD14 was absent (Fig 15 a, c). The 4 h peak of TNF after stimulation with *E. coli* O18K⁻ LPS was greatly diminished at high levels of anti-CD14 mAb for both the human MNL and THP-1 cells with enhancement for CD14. Statistical analysis by a one sided students t test showed that the difference between the response of TNF at 4 h by *E. coli* at low and high levels of anti-CD14 mAb was

Table 14: TNF production (IU/ml) after 4 h from human MNL and THP-1 cells with and without enhancement for CD14 after stimulation by *E. coli* O18K⁻ LPS (40 ng/ml), with *B. fragilis* NCTC 9343 LPS (160 ng/ml) present in excess at various time intervals. Each value is the mean of duplicate experiments performed with the same cell preparations.

	TNF	production	(IU/ml)
Time (h) after start of experiment in which *B. fragilis* NCTC 9343 LPS added to *E. coli* O18K⁻ LPS	Human MNL	THP-1 cells with enhancement for CD14	THP-1 cells without enhancement for CD14
0	52.98	75.65	89.3
1	51.48	46.21	91.55
2	62.42	85.95	108.21
3	87.63	90.21	120.39
E. coli O18K⁻ alone	140.66	102.7	170.02

Fig 14: Inhibition of TNF production after 4 h by a CD14 mAb from a) human mononuclear leukocytes, b) THP-1 cells without enhancement for CD14 and c) THP-1 cells with enhancement for CD14 after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (●), *B. fragilis* NCTC 9343 (▲) and CD14 monoclonal antibody alone (---). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.

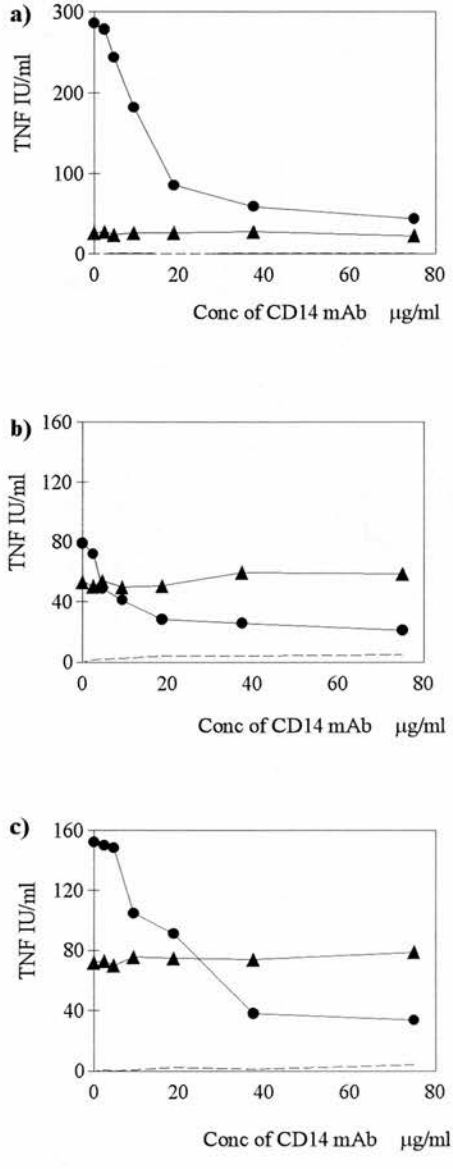
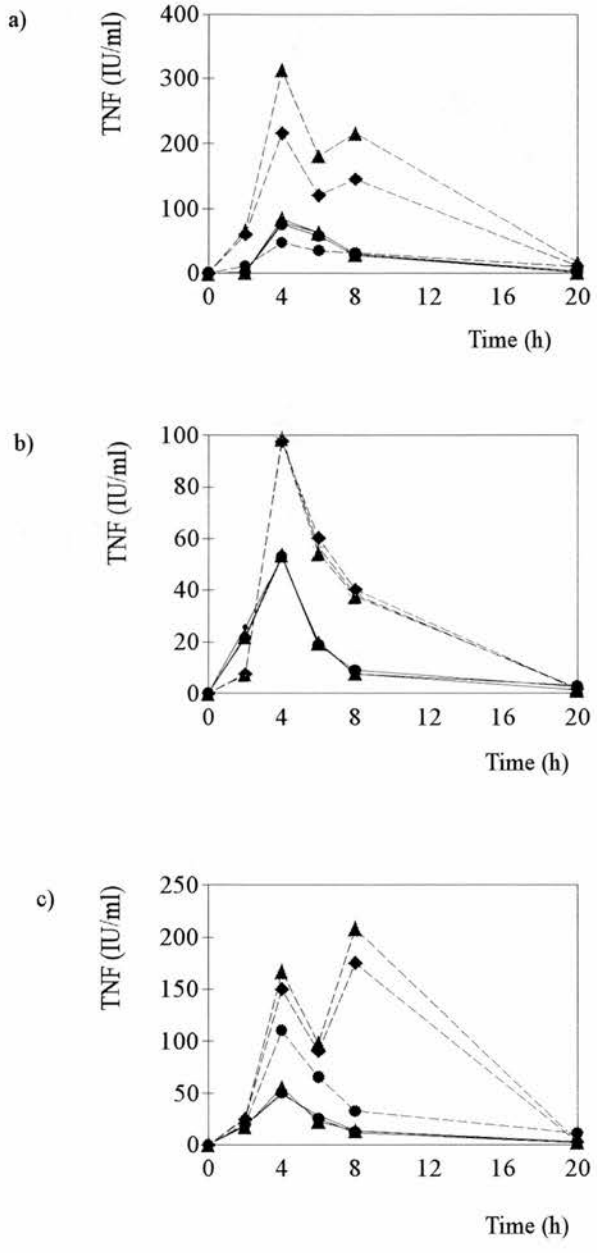


Fig 15: Time course of TNF (IU/ml) production after inhibition by (●) 75 μg/ml, (◆) 2.34 μg/ml and (▲) no anti-CD14 mAb following stimulation of a) human MNL, b) THP-1 cells without enhancement for CD14 and c) THP-1 cells with enhancement for CD14 with *E. coli* O18 K⁻ (----) LPS and *B. fragilis* NCTC 9343 (___) LPS (50 ng/ml). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.



significant for MNL where p=0.021. The same results analysed by the Mann Whitney test to compare medians also showed that the reduction in TNF for MNL was significant with p=0.04, where p<0.05 is significant. Results for the THP-1 cells with enhancement for CD14 were approaching significance where p=0.054 with the student t test and p=0.095 with the Mann Whitney test. The CD14 mAb had no effect on the kinetics of *Bacteroides* LPS induced TNF release in all cell populations or on the kinetics of TNF release in THP-1 cells without enhancement for CD14 (Fig 15 b).

3.10.12 Inhibition of TNF Production by Anti-*Bacteroides* mAbs

The effect of anti-*Bacteroides* mAbs in inhibiting TNF production was examined. In all cell populations, the presence of mAb 6g3 did not effect the production of TNF stimulated by any of the LPSs tested (Table 15). The mAb 4CS only slightly inhibited the production of TNF by *E. coli* O18K$^-$ LPS in human MNL but not in other cell populations. In all cell populations the presence of 3C8 inhibited the production of TNF stimulated by *B. fragilis* NCTC 9343 and *B. caccae* MPRL 1555 LPS but not that of *E. coli* O18K$^-$ LPS. The presence of 5A12, 3D7 and 1A4 inhibited the production of TNF stimulated by *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 LPS. The presence 5A12 caused the most marked inhibition of TNF production stimulated by *B. fragilis* NCTC 9343 LPS. There was low level background stimulation of TNF for each mAb. Inhibition of TNF production stimulated by *B. vulgatus* MPRL 1985 LPS was not seen due the low amount of TNF produced by this LPS and the background level of TNF produced by the mAbs alone.

3.10.13 Effect of TNF Production by *E. coli* O18K- LPS with *B. fragilis* NCTC 9343 LPS Present in Excess Together With an Anti-*Bacteroides* mAb

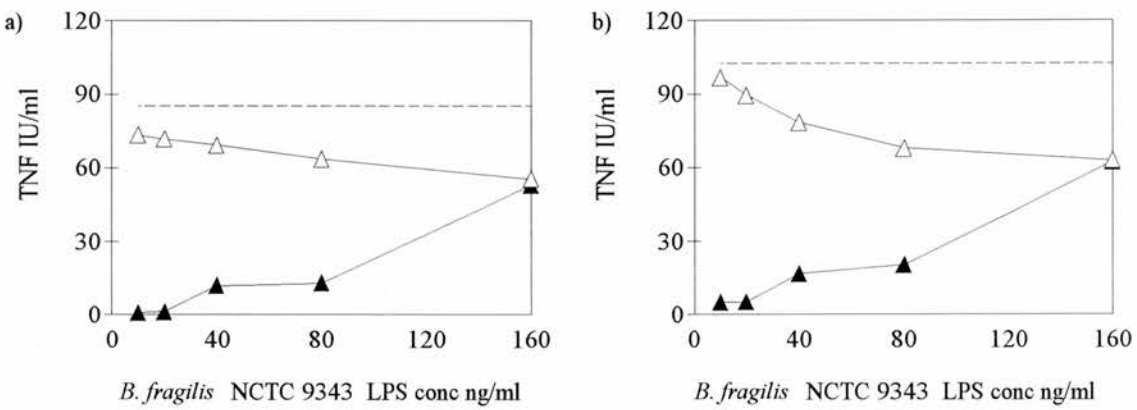
In the absence of an anti-*Bacteroides* mAb, there was a lowering and masking effect of TNF production from *E. coli* O18K$^-$ LPS when *B. fragilis* NCTC 9343 LPS was present in excess (Fig 16 b) as previously seen in Fig 12. In the presence of a 1:20

Table 15: Inhibition of TNF production (IU/ml) after 4 h by a 1:20 dilution of anti-*Bacteroides* mAbs from human MNL, THP-1 cells with and without enhancement for CD14 after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻, *B. fragilis* NCTC 9343, *B. caccae* MPRL 1555 and *B. vulgatus* MPRL 1985. Each value is the mean of duplicate experiments performed with the same cell population.

		TNF induced by mAb at 1:20 dilution						
Sample	Cell population	no mAb	5A12	1A4	4CS	3D7	6G3	3C8
E. coli O18K⁻	MNL	87.76	41.59	44.10	61.78	50.36	84.21	87.52
	THP-1 -	86.3	73.21	70.27	80.13	77.31	82.47	82.73
	THP-1 +	187.5	161.0	160.32	170.01	151.5	187.32	186.3
B. fragilis NCTC 9343	MNL	37.63	10.46	10.36	35.21	10.15	34.65	18.47
	THP-1 -	52.58	23.13	33.37	49.08	30.94	52.0	41.06
	THP-1 +	50.61	25.93	40.84	48.92	32.85	50.06	39.74
B. caccae MPRL 1555	MNL	24.94	20.85	23.92	18.85	16.69	18.57	16.70
	THP-1 -	30.52	...	...	...	...	...	22.77
	THP-1 +	26.87	...	...	...	...	...	18.77
B. vulgatus MPRL 1985	MNL	2.76	1.89	3.31	2.09	2.18	2.94	2.97
	THP-1 -	17.4	...	...	...	...	...	16.52
	THP-1 +	22.21	...	...	...	...	...	21.64
mAb only	MNL	0	0.37	0.641	0.92	1.71	1.07	0.98
	THP-1 -	0	0.2	1.33	0.53	0.0	0.21	0.90
	THP-1 +	0	0.01	0.02	0.14	0.57	0.98	1.80

Where MNL= human MNL, THP-1 - = THP-1 cells without enhancement for CD14, THP-1 + = THP-1 cells with enhancement for CD14 and ... = not done.

Fig 16: TNF production (IU/ml) after 4 h by human MNL after stimulation with varying amounts of *B. fragilis* NCTC 9343 LPS (▲) and varying amounts of *B. fragilis* NCTC 9343 LPS together with a constant amount of *E. coli* O18K⁻ (40 ng/ml) (Δ) LPS in the a) presence or b) absence of a 1:20 dilution of 5A12 anti-*Bacteroides* mAb. The maximal amount of TNF produced by *E. coli* O18K⁻ LPS alone shown by (----). Each point is the mean of duplicate experiments performed with the same cell preparations.



dilution of 5A12 mAb, the amount of TNF produced was lowered and the same pattern of results seen (Fig 16 a).

3.10.14 Inhibition of TNF Production by an Anti-TNF mAb

To ensure killing of L929 cells was due to TNF and not other factors, a dilution of anti-TNF mAb was incubated with cell culture supernates (derived after 4 h from human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ and *B. fragilis* NCTC 9343) prior to addition to L929 cells. The inhibition of TNF activity for both LPSs was nearly 100% for dilutions of anti-TNF mAb tested (Table 16).

Table 16: Inhibition of TNF production (IU/ml) after 4 h by an anti-TNF mAb from human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ and *B. fragilis* NCTC 9343.

	TNF	production	IU/ml
LPS	no mAb	+ 1:100 dilution TNF mAb	+ 1:1000 dilution TNF mAb
E. coli O18K⁻	36.00	0.21	0.69
B. fragilis NCTC 9343	78.60	0.14	0.68

3.11 REACTIVITY OF LPS ON THE BASIS OF KDO CONCENTRATION

All the assays in sections 3.9 and 3.10 have been done on a weight for weight basis. However, as endotoxicity is likely to be dependent on lipid A concentration it was decided to re-evaluate some of the results of TNF induction and LAL activity relative to KDO concentration in an attempt to compare lipid A levels - making the assumption, which could well be false, that lipid A concentration is proportional to KDO concentration in all species.

Measured TNF concentration IU/ng KDO from human MNL and endotoxic activity in LAL IU/ng KDO after stimulation with selected *Bacteroides* and *E. coli* O18K⁻ LPS is presented in Table 17. For measured TNF concentrations, this shows that previously low activities seen on a weight for weight basis (Table 13) were due in part to less KDO being present. However, LAL activity for PCP extracted LPS is still low when the results are represented per ng KDO.

Table 17: Measured TNF concentration IU/ng KDO from human MNL and endotoxic activity in LAL IU/ng KDO after stimulation with selected *Bacteroides* and *E. coli* O18K⁻ LPS

LPS Sample	Extraction Method	TNF Stimulation IU/ng KDO	LAL Activity EU/ng KDO
B. fragilis NCTC 9343 VT	PW	59.38	1583.08
	PCP	7.16	10.69
	Triton	17.3	2784.93
B. fragilis NCTC 9343 PPY	PW	46.11	1191.11
	PCP	35.11	8.44
	Triton	4.90	693.24
B. fragilis MPRL 1504	PW	15.85	5985.54
	PCP	17.42	37.38
	Triton	5.38	3846.99
B. vulgatus MPRL 1985	PW	19.11	115.48
	PCP	3.41	7.93
	Triton	3.00	84.67
B. thetaiotaomicron MPRL 1720	PW	...	...
	PCP	1.55	42.61
	Triton	8.64	867.91
B. ovatus MPRL 1709	PW	30.37	14.98
	PCP	25.89	4.68
	Triton	10.43	385.5
E. coli O18K⁻ aerobically grown	PW	502.16	572.32
E. coli O18K⁻ anaerobically grown	PW	485.60	261.15

See Table 3 for abbreviations.

3.12 CAPSULAR POLYSACCHARIDE INDUCED TNF PRODUCTION

3.12.1 Effect of Amount of Capsular Polysaccharide on TNF Production

The effect of varying amounts of high and low molecular weight (mwt) capsular polysaccharide (CP) from *B. fragilis* NCTC 9343 in inducing TNF production from human MNL was examined compared to LPS controls (Table 18). Comparing TNF production at a sample size of 50 ng/ml, *E. coli* O18K⁻ LPS induced the most TNF, with *B. fragilis* NCTC 9343 LPS inducing four-fold less TNF and low mwt CP inducing approximately fifty three-fold less TNF than *E. coli* O18K⁻ LPS. At this level high mwt CP failed to induce any TNF production. TNF production by 500-5000 ng/ml of low mwt CP was comparable to that produced by 50 ng/ml of *B. fragilis* NCTC 9343 LPS.

Table 18: Measured TNF concentration (IU/ml) from human MNL after stimulation for 4 h with varying amounts of high and low molecular weight capsular polysaccharide from *B. fragilis* NCTC 9343 and LPS from *B. fragilis* NCTC 9343 and *E. coli* O18K⁻. Each value is the mean of duplicate experiments performed with the same cell preparations.

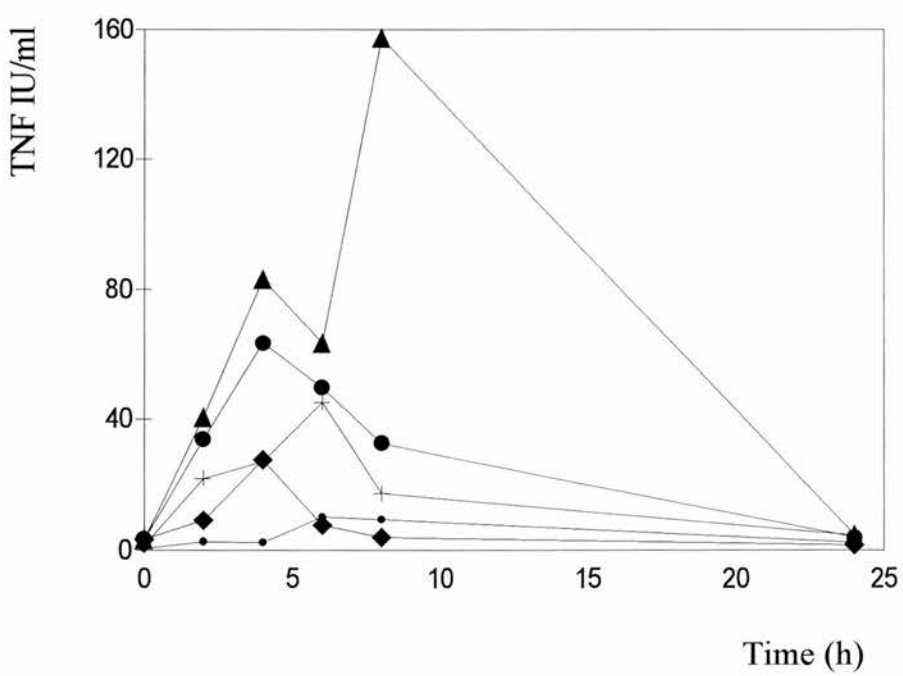
	TNF		Production	IU/ml
Sample ng/ml	High mwt CP	Low mwt CP	*B.fragilis* NCTC 9343 LPS	*E. coli* O18K⁻ LPS
50000	1.421	36.72	...	...
5000	0	34.49	...	...
500	0	14.64	...	...
50	0	1.69	21.44	90.17
5	0	0.06	7.21	45.25
0.5	0	0	...	...
0.05	0	0	...	...

Where mwt = molecular weight, CP= capsular polysaccharide and ... = not done. See Table 3 for other abbreviations.

3.12.2 Time course of TNF Production

Levels of TNF production by human MNL were measured every 2 h after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343, and with high/low mwt CP (500 ng/ml) from *B. fragilis* NCTC 9343 (Fig 17). As previously seen in Fig 10 a peak of TNF production at 4 h was seen after stimulation with all LPSs tested, with a second peak at 8 h seen for *E. coli* O18K$^-$ LPS only. A peak of TNF production at 6 h was seen after stimulation with both high and low mwt CP from *B. fragilis* NCTC 9343.

Fig 17: Time course of TNF production (IU/ml) from human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ (▲), *B. fragilis* NCTC 9343 (●) and *B. vulgatus* MPRL 1985 (◆); and 500 ng/ml of high mwt CP (•) and low mwt CP (+) from *B. fragilis* NCTC 9343. Each point is the mean of duplicate experiments performed with the same cell preparations.



3.12.3 Effect of TNF Production by *E. coli* O18K- LPS With *B. fragilis* NCTC 9343 Capsular Polysaccharide Present in Excess

When LPS from *B. fragilis* NCTC 9343 was in excess of a constant amount of *E. coli* O18K⁻ LPS (50 ng/ml) in human MNL, TNF production was comparable to that of *B. fragilis* NCTC 9343 LPS alone and not that of *E. coli* O18K⁻ LPS alone (Table 19). This lowering and masking effect of TNF production from *E. coli* O18K⁻ LPS previously described in section 3.10.7 was enhanced when an excess of low mwt CP from *B. fragilis* NCTC 9343 was present, and reduced when an excess of high mwt CP from *B. fragilis* NCTC 9343 was present (Table 19).

Table 19: Effect of TNF production (IU/ml) from human MNL by *E. coli* O18K⁻ LPS with *B. fragilis* NCTC 9343 LPS and capsular polysaccharide present in excess. Each value is the mean of duplicate experiments performed with same cell preparations.

Sample	TNF production (IU/ml)
E. coli O18K⁻ LPS (50 ng/ml)	151.4
B. fragilis NCTC 9343 LPS (160 ng/ml)	58.72
B. fragilis NCTC 9343 high mwt CP (500 ng/ml)	3.10
B. fragilis NCTC 9343 low mwt CP (500 ng/ml)	27.6
E. coli O18K⁻ LPS (50 ng/ml) + *B. fragilis* NCTC 9343 LPS (160 ng/ml)	62.76
E. coli O18K⁻ LPS (50 ng/ml) + *B. fragilis* NCTC 9343 high mwt CP (500 ng/ml)	111.5
E. coli O18K⁻ LPS (50 ng/ml)+ *B. fragilis* NCTC 9343 low mwt CP (500 ng/ml)	57.28

Where mwt = molecular weight and CP= capsular polysaccharide. See Table 3 for other abbreviations.

3.13 LPS INDUCED SECRETION OF INTERLEUKIN-8

3.13.1 IL-8 Production by *Bacteroides* LPSs Extracted by Three Different Methods

E. coli O18K$^-$ LPS stimulated the most IL-8 production with no difference in IL-8 production between *E. coli* O18K$^-$ LPS grown aerobically or anaerobically (Table 20). On a weight for weight basis, PW-LPS from *Bacteroides* species stimulated the most IL-8 production and PCP-LPS the least. Comparing PW-LPS, *Bacteroides* LPS induced IL-8 at a magnitude much less than *E. coli* O18K$^-$, with the most active (*B. fragilis* NCTC 9343 VT) being approximately three times less active than *E. coli* O18K$^-$ and the least active *B. thetaiotaomicron* MPRL 1720 being 13 times less active than *E. coli* O18K$^-$. Matched TNF production results are shown in Table 13.

3.13.2 Time course of IL-8 Production

Levels of IL-8 by human MNL were measured at hourly intervals after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 (Fig 18 a). The response produced by the *E. coli* O18K$^-$ LPS showed a uniform rise and fall, peaking at 6-7 hours. The *B. fragilis* NCTC 9343 LPS showed peaks of IL-8 production at 6, 9 and 12 hours. Matched TNF production results are shown in Fig 10 a.

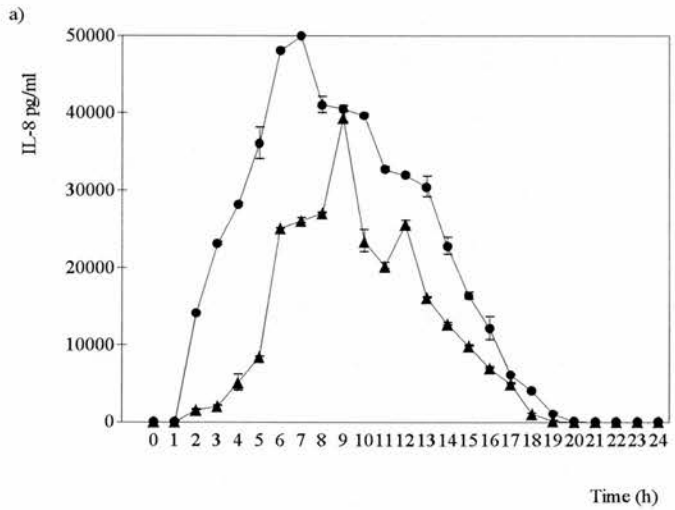
IL-8 production by sampling every two hours in THP-1 cells with and without enhancement for CD14 was followed (Fig 18 b, c). In THP-1 cells without enhancement for CD14 (Fig 18 b) IL-8 production occurred on a much smaller magnitude. *E. coli* O18K$^-$ LPS was the least active producing one peak of IL-8 at 4 hours and *B. vulgatus* MPRL 1985 LPS the most active. Both *Bacteroides* LPS produced peaks of IL-8 at both 4 and 8 hours. In THP-1 cells with enhancement for CD14 (Fig 18 c) IL-8 production occurred on a greater magnitude with levels of IL-8 produced comparable to those found in human MNL. *E. coli* O18K$^-$ LPS produced

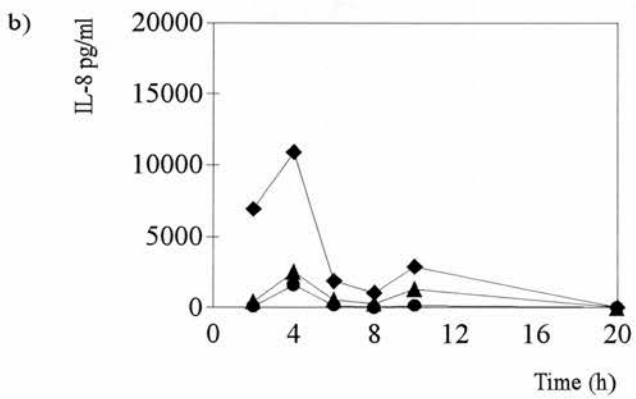
Table 20: Measured IL-8 concentration pg/ml from human MNL after 4 h stimulation with native form LPS (50 ng/ml) from *E. coli* O18K⁻ and *Bacteroides* species extracted by three different methods. Each value is the mean of duplicates from one experiment.

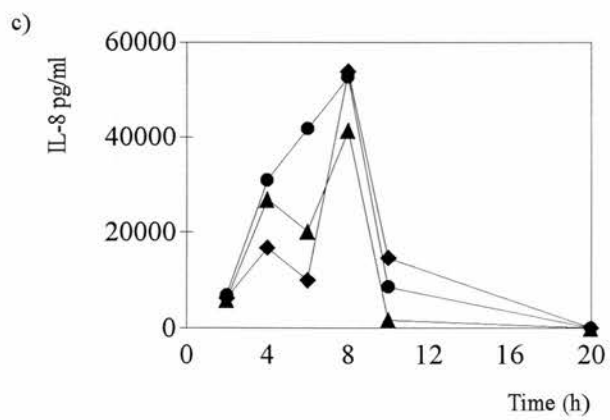
Native LPS Sample	Extraction Method	IL-8 Production pg/ml
B. fragilis NCTC 9343 VT	PW	9320
	PCP	3006
	Triton	1629
B. fragilis NCTC 9343 PPY	PW	4695
	PCP	1541
	Triton	2189
B. fragilis MPRL 1504	PW	5102
	PCP	2294
	Triton	4401
B. vulgatus MPRL 1985	PW	3027
	PCP	1552
	Triton	3216
B. thetaiotaomicron MPRL 1720	PW	2034
	PCP	1383
	Triton	1592
B. caccae MPRL 1555	PW	5084
	PCP	1212
	Triton	2764
B. uniformis MPRL 1721	PW	5343
	PCP	2339
	Triton	3863
B. ovatus MPRL 1709	PW	7088
	PCP	1522
	Triton	3122
E. coli O18 K⁻ aerobically grown	PW	29784
E. coli O18K⁻ anaerobically grown	PW	26360

See Table 13 for matched TNF production results. See Table 3 for abbreviations.

Fig 18: Time course of IL-8 production (pg/ml) by a) human MNL, b) THP-1 cells without enhancement for CD14 and c) THP-1 cells with enhancement for CD14 after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (●), *B. fragilis* NCTC 9343 (▲) and *B. vulgatus* MPRL 1985 (◆). Each point is the mean of duplicates from one experiment. Note different scales on y axes.







one peak of IL-8 at 8 hours and both *Bacteroides* LPSs produced peaks of IL-8 at both 4 and 8 hours.

3.13.3 Effect of IL-8 Production by *E. coli* O18K⁻ LPS with *B. fragilis* NCTC 9343 LPS Present in Excess

When LPS from *B. fragilis* NCTC 9343 was in excess of a constant amount of *E. coli* O18K⁻ LPS (40 ng/ml) in human MNL, IL-8 production was comparable to that of *B. fragilis* NCTC 9343 LPS alone and not that of *E. coli* O18K⁻ alone (Fig 19). There appeared to be a lowering and masking effect of IL-8 production from *E. coli* O18K⁻ LPS when LPS from *B. fragilis* NCTC 9343 was present in excess. Matched TNF production results are shown in Fig 12 a.

3.13.4 Inhibition of IL-8 Production by An Anti-CD14 mAb

The effect of an anti-CD14 mAb in inhibiting IL-8 production was examined (Fig 20). In all cell populations tested, the production of IL-8 stimulated by both *E. coli* O18K⁻ and *B. fragilis* NCTC 9343 LPS was slightly inhibited as the concentration of CD14 mAb increased. Matched TNF production results are shown in Fig 14.

3.13.5 Inhibition of IL-8 Production by Anti-*Bacteroides* mAbs

All anti-*Bacteroides* mAb had no effect in inhibiting IL-8 production from human MNL after stimulation with all LPSs tested (Table 21). Matched TNF production results are shown in Table 15.

Fig 19: IL-8 production (pg/ml) after 4 h by human MNL after stimulation with varying amounts of LPS from *B. fragilis* NCTC 9343 (▲), varying amounts of *B. fragilis* NCTC 9343 LPS together with a constant amount of *E. coli* O18K⁻ (40 ng/ml) (Δ). Maximum amount of IL-8 produced by *E. coli* O18K⁻ alone shown by (---). Each point is the mean of duplicate experiments performed with the same cell preparations.

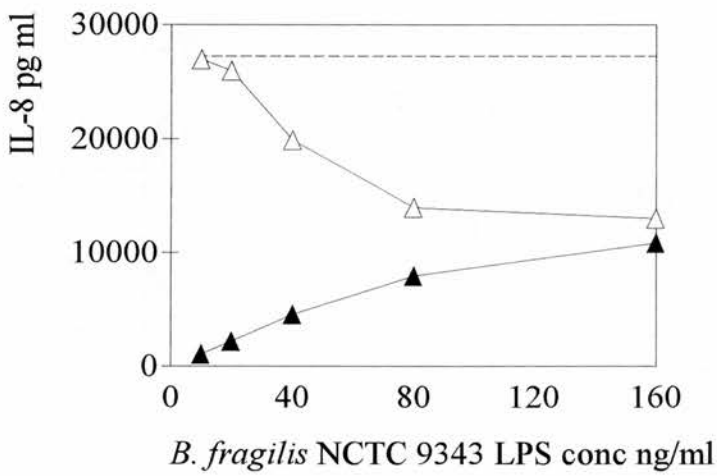


Fig 20: Inhibition of IL-8 production (pg/ml) after 4 h by an anti-CD14 mAb from a) human mononuclear leukocytes, b) THP-1 cells without enhancement for CD14, and c) THP-1 cells with enhancement for CD14, after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (●), *B. fragilis* NCTC 9343 (▲) and CD14 monoclonal antibody alone (---). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on y axes.

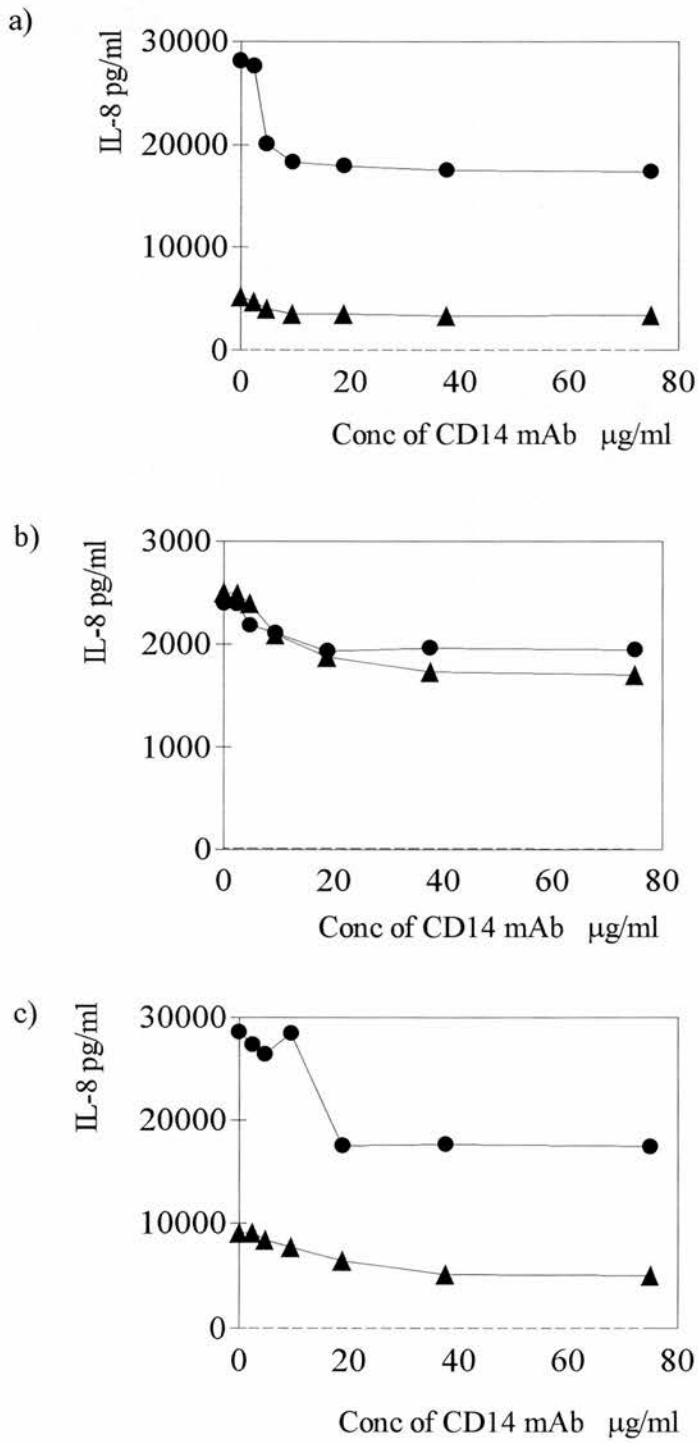


Table 21: Inhibition of IL-8 production (pg/ml) after 4 h by a 1:20 dilution of anti-*Bacteroides* mAbs from human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$, *B. fragilis* NCTC 9343, *B. caccae* MPRL 1555 and *B. vulgatus* MPRL 1985. Each value is the mean of duplicate experiments performed with the same cell population.

	IL-8 induced by mAb at 1:20 dilution						
Sample	no mAb	5A12	1A4	4CS	3D7	6G3	3C8
E. coli O18K-	25684	24920	25650	24539	28228	26312	27851
B. fragilis NCTC 9343	9306	9495	9082	8848	9719	9063	9333
B. caccae MPRL 1555	8178	8655	8363	8577	8898	7898	8504
B. vulgatus MPRL 1985	4314	4325	4598	4804	4578	4892	4801
mAb only	0	202	314	250	302	119	344

3.14 CAPSULAR POLYSACCHARIDE INDUCTION OF INTERLEUKIN-8

Levels of IL-8 by human MNL were measured every 2 h after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343; and low mwt CP (500 ng/ml) and high mwt CP (500 ng/ml) from *B. fragilis* NCTC 9343 (Fig 21). As seen in Fig 18, *E. coli* O18K$^-$ LPS induced one peak of IL-8 at 6 h whereas *B. fragilis* NCTC 9343 LPS induced two peaks of IL-8 at 6 and 10 h. Low mwt CP induced one peak of IL-8 at 10 h and high mwt CP induced increasing quantities of IL-8 over time with maximum levels observed after 24 h stimulation. Maximum levels of IL-8 produced by CPs and LPSs are comparable.

3.15 LPS INDUCTION OF INTERLEUKIN-6

3.15.1 Effect of Amount of LPS on IL-6 Production

The effect of varying ng amounts of LPS on IL-6 production from human MNL was examined for *E. coli* O18K$^-$, *B. fragilis* NCTC 9343, *B. vulgatus* MPRL 1985, *B. caccae* MPRL 1555 and *B. thetaiotaomicron* MPRL 1720 (Fig 22). *E. coli* O18K$^-$ LPS produced more IL-6 than all *Bacteroides* LPSs for all amounts tested. The most active *Bacteroides* LPS (*B. fragilis* NCTC 9343) was approximately two-fold less active than *E. coli* O18K$^-$ LPS and the least active (*B. vulgatus* MPRL 1985) being approximately seven-fold less active.

3.15.2 Time course of IL-6 Production

IL-6 induction by LPSs from *Bacteroides* species and *E. coli* O18K$^-$ by sampling every two hours was examined in human MNL and in THP-1 cells with and without enhancement for CD14 (Fig 23). In all cell populations, all LPSs produced a single peak of IL-6 production at 6-8 hours. There was little, if any, difference in IL-6 produced between THP-1 cells with and without enhancement for CD14 for both *E. coli* O18K$^-$ LPS and *B. fragilis* NCTC 9343 LPS.

Fig 21: Time course of IL-8 production (pg/ml) by human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ (▲), *B. fragilis* NCTC 9343 (●) and 500 ng/ml of low mwt CP (◆) and high mwt CP (■) from *B. fragilis* NCTC 9343. Each point is the mean of duplicates from one experiment.

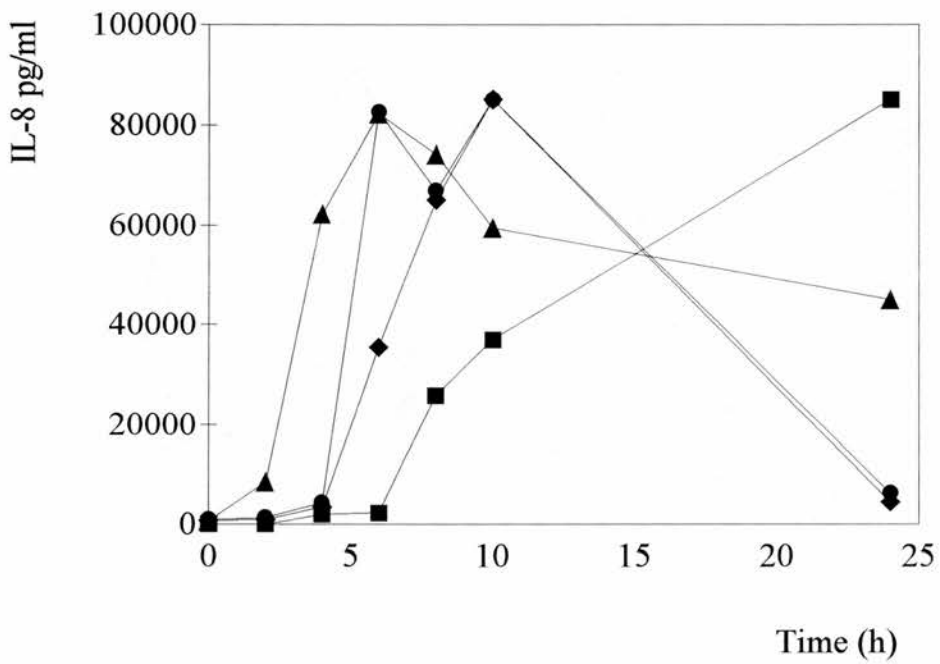


Fig 22: IL-6 production (pg/ml) from human MNL after stimulation for 4 h with LPS from *E. coli* O18K⁻ (▲), *B. fragilis* NCTC 9343 (●), *B. caccae* MPRL 1555 (◆), *B. vulgatus* MPRL 1985 (•) and *B. thetaiotaomicron* MPRL 1720 (+). Each point is the mean of duplicates from one experiment.

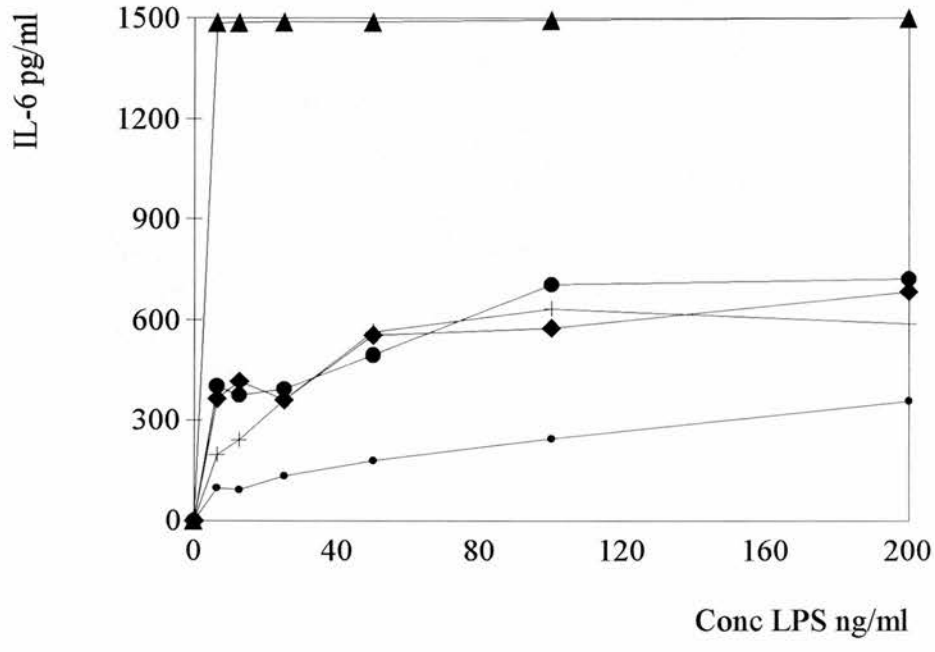
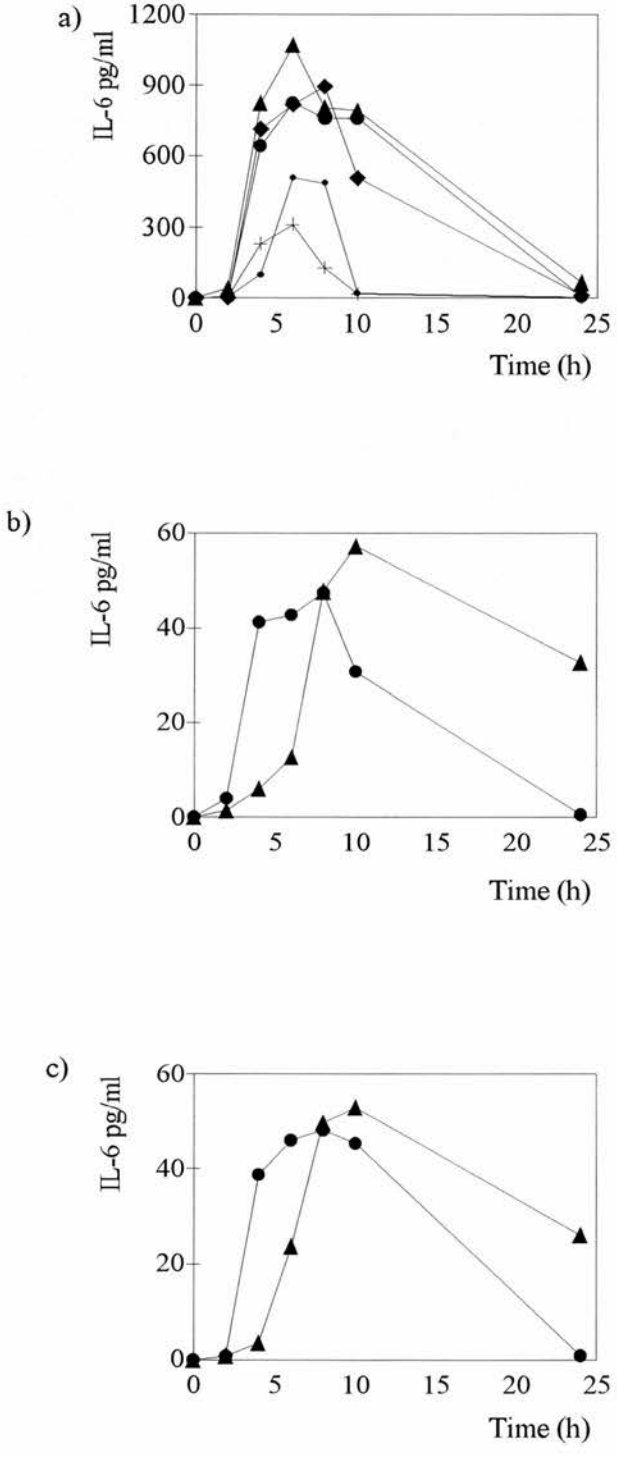


Fig 23: Time course of IL-6 production (pg/ml) by a) human MNL, b) THP-1 cells without enhancement for CD14 and c) THP-1 cells with enhancement for CD14 after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (▲), *B. fragilis* NCTC 9343 (●), *B. caccae* MPRL 1555 (◆), *B. vulgatus* MPRL 1985 (•) and *B. thetaiotaomicron* MPRL 1720 (+). Each point is the mean of duplicates from one experiment. Note different scales on y axes.



3.15.3 Effect of IL-6 Production by *E. coli* O18K⁻ LPS with *B. fragilis* NCTC 9343 LPS Present in Excess

When LPS from *B. fragilis* NCTC 9343 was in excess of a constant amount of *E. coli* O18K⁻ LPS (40 ng/ml) in human MNL. IL-6 production was comparable to that of *B. fragilis* NCTC 9343 LPS alone and not that of *E. coli* O18K⁻ alone (Fig 24). There appeared to be a lowering and masking effect of IL-6 production from *E. coli* O18K⁻ LPS when LPS from *B. fragilis* NCTC 9343 was present in excess.

3.15.4 Inhibition of IL-6 Production by an Anti-CD14 mAb

The anti-CD14 mAb did not inhibit IL-6 production in all cell populations tested after stimulation with both *E. coli* O18K⁻ and *B. fragilis* NCTC 9343 LPS (Fig 25).

3.16 LPS INDUCTION OF SOLUBLE p55 TNF RECEPTOR

Levels of soluble p55 TNF receptor (sTNFr) from human MNL was measured at two hourly intervals after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ and various *Bacteroides* species (Fig 26). Levels of p55 TNF receptor fell to zero at 4 h for all LPSs tested except *B. vulgatus* MPRL 1985 which fell to a minimum value at 6 h. The production of p55 sTNFr steadily rose for all LPSs (including that of the control which contained no LPS), to maximum levels observed by 24 h at the end of the experiment. At 24 h *E. coli* O18K⁻ LPS had induced the most p55 sTNFr and *B. vulgatus* the least.

Fig 24: IL-6 production (pg/ml) after LPS stimulation for 4 h by human MNL after stimulation with varying amounts of LPS from *B. fragilis* NCTC 9343 (▲), varying amounts of *B. fragilis* NCTC 9343 LPS together with a constant amount of *E. coli* O18K⁻ (40 ng/ml) (Δ). Maximum amount of IL-6 produced by *E. coli* O18K⁻ alone shown by (---). Each point is the mean of duplicates from one experiment.

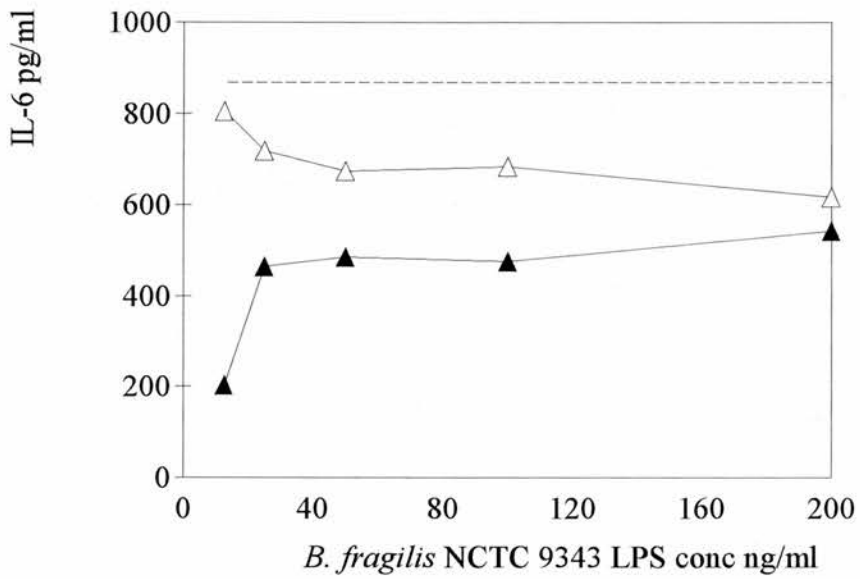


Fig 25: Inhibition of IL-6 production after 4 h by a CD14 mAb from a) human MNL, b) THP-1 cells without enhancement for CD14, and c) THP-1 cells with enhancement for CD14, after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (●) , *B. fragilis* NCTC 9343 (▲) and CD14 monoclonal antibody alone (---). Each point is the mean of duplicates from one experiment. Note different scales on y axes.

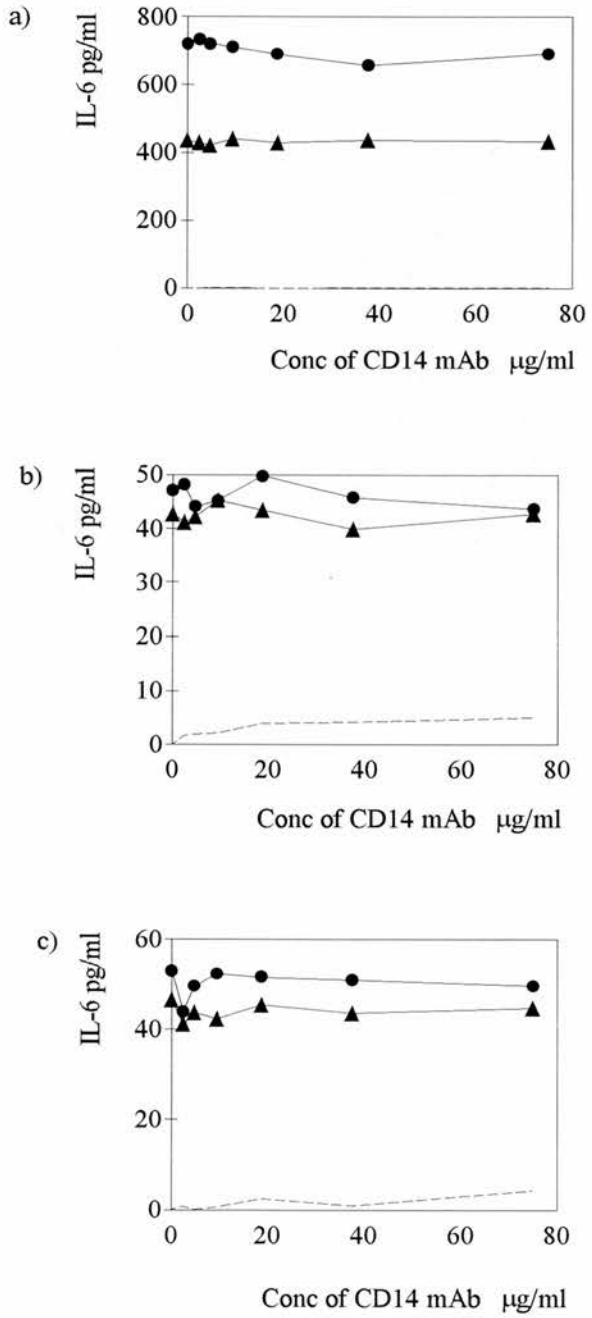
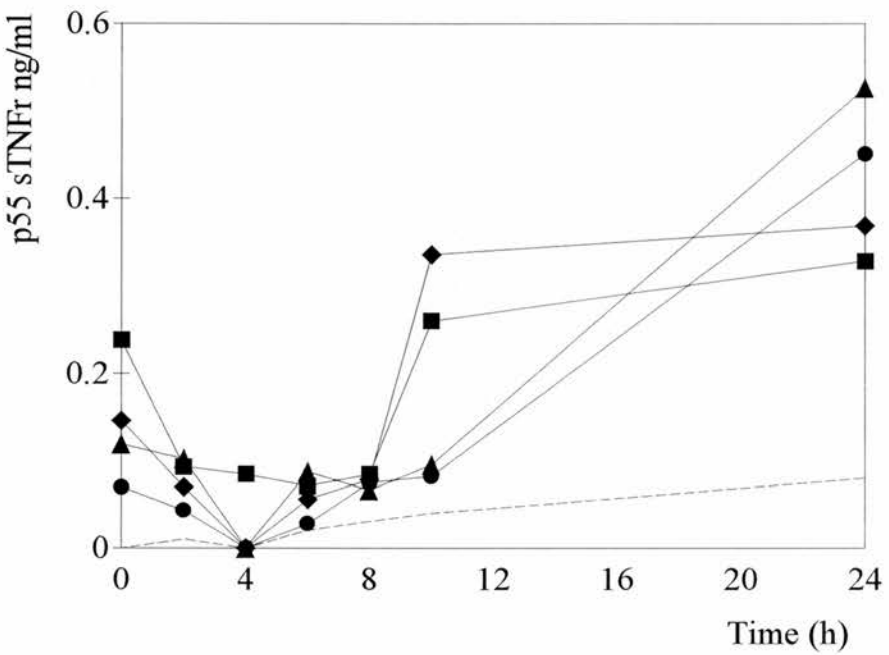


Fig 26: Time course of p55 soluble TNF receptor production (ng/ml) from human MNL after stimulation with LPS (50 ng/ml) from *E. coli* O18K⁻ (▲), *B. fragilis* NCTC 9343 (●), *B. caccae* MPRL 1555 (◆) and *B. vulgatus* MPRL 1985 (■). Amount produced by culture medium control shown by (---). Each point is the mean of duplicate experiments performed with the same cell preparations.



3.17 MITOGENICITY OF *BACTEROIDES* LPSs

3.17.1 Mitogenicity of *Bacteroides* LPSs to Mouse Spleen and Lymph Nodes

The mitogenicity of *Bacteroides* LPSs to mixed cell populations from mouse spleen and lymph nodes is shown in Table 22. Those LPSs which induced a value greater than the PF-H_2O negative control were considered to be mitogenic. By comparing the results obtained by 25 μg/ml of LPS, most mitogenicity was seen towards the spleen cell population. *B. vulgatus* MPRL 1985 LPS induced the most mitogenicity followed by *E. coli* O18K$^-$ LPS and *B. caccae* MPRL 1555 LPS, with *B. fragilis* NCTC 9343 LPS inducing the least effect.

3.17.2 Mitogenicity of *Bacteroides* LPSs and Capsular Polysaccharides to Mouse B Cells, T Cells and Mixed Spleen Cell Populations

In another experiment the mitogenicity of *Bacteroides* LPS and CPs from *B. fragilis* NCTC 9343 to mouse B cells, T cells as well as mixed cell populations of mouse spleen cells, is shown in Table 23. Control values were high in this instance but those LPSs or CPs which induced a value greater than the PF-H_2O negative control were considered to be mitogenic. To all cell populations *E. coli* O18K$^-$ LPS induced the most mitogenicity while *B. vulgatus* MPRL 1985 LPS induced more mitogenicity than *B. fragilis* NCTC 9343 LPS in all cell populations apart from the T cell population where *B. fragilis* NCTC 9343 LPS was more active than *B. vulgatus* MPRL 1985 LPS. In all cell populations high mwt CP was more mitogenic than low mwt CP. In mixed spleen cells and T cells the level of mitogenicity by high CP was comparable to that found by *E. coli* O18K$^-$ LPS.

Table 22 : Mitogenicity of *Bacteroides* LPSs to mouse spleen and lymph nodes

Sample	μg/ml	Counts per minute per 2 x 10^6 cells/ml Spleen	Counts per minute per 2 x 10^6 cells/ml Lymph nodes
Control (PF-H_2O)	-	3700	3400
PHA	5	11,100	11,633
E. coli O18K$^-$ LPS	25	13,333	4933
	10	5533	5767
	5	3233	4900
	1	4600	4767
B. fragilis NCTC 9343 LPS	25	5800	3800
	10	4633	3567
	5	5333	3406
	1	3533	5100
B. vulgatus MPRL 1985 LPS	25	21,333	6200
	10	10,500	5133
	5	8833	4200
	1	6233	5200
B. caccae MPRL 1555 LPS	25	9567	4933
	10	7667	4767
	5	8333	5100
	1	6333	6400

Where PHA= phytohaemagglutinin. Each value is mean of triplicates from a single experiment. Results repeated on at least two occasions.

Table 23: Mitogenicity of *Bacteroides* LPSs and capsular polysaccharide from *B. fragilis* NCTC 9343 to mouse B cell, T cell and mixed spleen cell populations

Sample (5 μg/ml)	Counts per min per 2 x 10^6 cells/ml Mixed Spleen Cells	Counts per min per 2 x 10^6 cells/ml B Cells	Counts per min per 2 x 10^6 cells/ml T Cells
Control (PF-H_2O)	18,799	8817	17,662
PHA	31,397	14,747	35,681
E. coli O18K$^-$ LPS	34,734	24,641	31,156
B. fragilis NCTC 9343 LPS	24,090	19,863	14,446
B. vulgatus MPRL 1985 LPS	25,213	21,917	22,962
High mwt CP	33,852	18,082	25,170
Low mwt CP	27,987	15,195	28,453

Where High mwt CP= High molecular weight capsular polysaccharide from *B. fragilis* NCTC 9343, Low mwt CP= Low molecular weight capsular polysaccharide from *B. fragilis* NCTC 9343 and PHA= phytohaemagglutinin. Each value is the mean of triplicates from a single experiment.

3.18 FLOW CYTOMETRIC ANALYSIS

3.18.1 Analysis of CD14 Positive Cells

To confirm the results shown in sections 3.10 and 3.11, the binding of an anti-CD14 mAb to human MNL and THP-1 cells with/without enhancement for CD14 was examined and the results illustrated in Table 24. The binding of the anti-CD14 mAb to THP-1 cells without enhancement for CD14 was low and increased three to four-fold in THP-1 cells with enhancement for CD14. The percentage of cells exhibiting binding of an anti-CD14 mAb to human MNL was at approximately the same level as THP-1 cells with enhancement for CD14.

3.18.2 Analysis of Mouse B Cell, T Cell and Mixed Spleen Cell Populations

In order to ensure homogeneous cell populations had been prepared for mitogenicity experiments (section 3.17.2) the binding of an anti-CD3 mAb to cells was examined and the results illustrated in Table 25. CD3 is a cell surface antigen present on T cells only. Those cells excluded and termed lymphocytes and macrophages are shown in Fig 27. No significant difference in percentage fluorescence with and without the presence of the CD3 mAb was seen and the binding to the macrophage population was also high, suggesting that non-specific binding of the FITC labelled mAb had occurred.

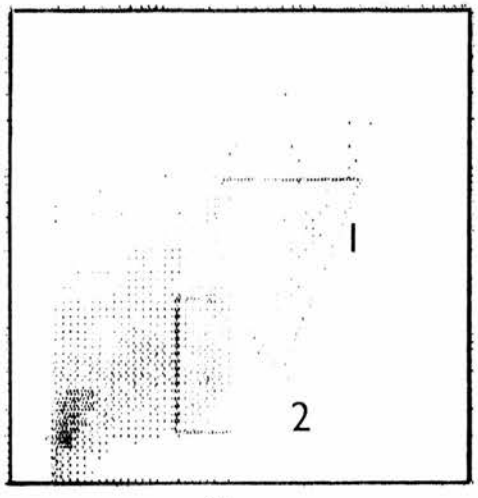
Table 24: Binding of an anti-CD14 mAb to human MNL and THP-1 cells with /without enhancement for CD14

Cell population	CD14 mAb µg/ml	% of 5000 cells exhibiting fluorescence
THP-1 cells without enhancement for CD14	0	0.95
	750	5.3
	375	4.5
THP-1 cells with enhancement for CD14	0	0.13
	750	17.5
	375	16.8
Human MNL	0	2.18
	750	16.8
	375	15.4

Table 25: Binding of an anti-CD3 mAb to mouse mixed spleen cell, B cell and T cell populations

Cell population	Anti-CD3 mAb	% fluorescence Lymphocytes	% fluorescence Macrophages
Spleen mix	+	45	74
	-	42	72
B cells	+	85	79
	-	85	85
T cells	+	50	85
	-	52	42

Fig 27: A representation of lymphocyte[1] and macrophage[2] cell populations from mixed mouse spleen cells



3.19 THE EFFECT ON THE CH_{50} OF HUMAN SERUM IN THE PRESENCE OF *BACTEROIDES* LPSs

The effect on the CH_{50} (units/ml) of human serum by LPSs from *Bacteroides* species and *E. coli* O18K$^-$ extracted by different methods was examined and the results illustrated in Table 26. Comparing the different types of LPSs extracted from *B. fragilis* NCTC 9343, PW-LPS induced the greatest increase in CH_{50} and PCP-LPS the least. Comparing the PW-LPSs, *E. coli* O18K$^-$ LPS induced the greatest increase in CH_{50} of human serum by approximately two-fold. LPS from *B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720 did not alter the CH_{50} of human serum.

When *B. fragilis* NCTC 9343 LPS was present together and in excess of *E. coli* O18K$^-$ LPS, the CH_{50} value of the serum was closer to that produced by *B. fragilis* NCTC 9343 LPS alone and not *E. coli* O18K$^-$ LPS alone (Table 27).

3.20 BINDING ACTIVITY OF *BACTEROIDES* mAbs TO LPSs

The binding activities in ELISA of anti-*Bacteroides* mAbs to various *Bacteroides* LPSs and *E. coli* O18K$^-$ LPS are shown in Table 28. The anti-*Bacteroides* LPSs did not bind well to the *Bacteroides* LPSs and *E. coli* O18K$^-$ LPS. The mAbs 4CS, 3C8 1A4 and 3D7 bound with the greatest affinity to *B. fragilis* NCTC 9343 LPS. The mAb 5A12 bound with greatest affinity to *E. coli* O18K$^-$ LPS and the mAb 6G3 did not bind significantly to any LPS tested. There was no non-specific binding to the uncoated plastic wells for any of the mAbs tested.

Table 26: Effect on CH_{50} (units/ml) of human serum by LPSs (100 ng/ml) from *Bacteroides* species and *E. coli* O18K⁻ extracted by different methods

LPS	Extraction method	CH_{50} units/ml of serum
Control (serum only)	-	60.25
E. coli O18K⁻	PW	127.33
B. fragilis NCTC 9343	PW	91.98
B. fragilis NCTC 9343	PCP	65.86
B. fragilis NCTC 9343	Triton	82.04
B. caccae MPRL 1555	PW	89.81
B. vulgatus MPRL 1985	PW	58.43
B. thetaiotaomicron MPRL 1720	PW	62.73

Table 27: Effect on CH_{50} (units/ml) of human serum by *E. coli* O18K⁻ LPS with *B. fragilis* NCTC 9343 LPS present in excess

Sample	CH_{50} units/ml of serum
Control (serum only)	63.24
E. coli O18K⁻ LPS (100 ng/ml)	118.2
B. fragilis NCTC 9343 LPS (100 ng/ml)	59.04
B. fragilis NCTC 9343 LPS (200 ng/ml)	108.79
E. coli O18K⁻ LPS (100 ng/ml) + *B. fragilis* NCTC 9343 LPS (100 ng/ml)	100.00
E. coli O18K⁻ LPS (100 ng/ml) + *B. fragilis* NCTC 9343 LPS (200 ng/ml)	72.32

For Tables 26 and 27, each value is the mean of triplicates from a single experiment. Results repeated on two occasions. See Table 3 for other abbreviations.

Table 28: Binding activities in ELISA of anti-*Bacteroides* mAbs (1:5 dilutions) to various *Bacteroides* LPSs and *E. coli* O18K⁻ LPS

	Mean OD 405 nm					
LPS	4CS	3C8	1A4	5A12	3D7	6G3
E. coli O18K⁻	0.124	0.133	0.123	0.130	0.011	0.031
B. fragilis NCTC 9343	0.385	0.227	0.267	0.109	0.110	0.011
B. vulgatus MPRL 1985	0.108	0.026	0.137	0.096	0.007	0.018
B. caccae MPRL 1555	0.086	0.023	0.106	0.104	0.004	0.024
B. thetaiotaomicron MPRL 1720	0.114	0.022	0.148	0.120	0.005	0.016
Control (uncoated)	0.021	0.022	0.019	0.024	0.006	0.021

Each value is the mean of triplicates from a single experiment. Results repeated on two occasions.

Discussion

4.1 DIFFERENCES IN BIOLOGICAL ACTIVITY BETWEEN EXTRACTION METHODS

Bacteroides LPS has been disregarded by many authors as being much less endotoxic than enterobacterial LPS (Lindberg *et al* 1990, Weintraub *et al* 1985), but this study has shown that the biological activity of *Bacteroides* LPS was dependent on the extraction method employed. The three methods were selected in an attempt to produce products of different chemical composition. The classical PW method was likely to select for higher molecular mass, less hydrophobic material; the PCP method for rough-form, more hydrophobic material; while the Triton-Mg^{2+} method was chosen as a milder, less selective method. The PW method produced LPS with the highest biological activity and the PCP and the Triton methods producing LPS with low biological activities. Which extracted LPS type, if any, reflects that *in vivo* remains to be defined.

In a mouse lethality model, the results obtained (Table 7) agree with previous studies (Lindberg *et al* 1990) that *Bacteroides* LPS is approximately 5000-fold less active than LPS from *E. coli.* In past studies (Lindberg *et al* 1990, Loppnow *et al* 1989, Fujiwara *et al* 1988) *Bacteroides* LPS has been quoted as being 100 to 1000-fold less biologically active *in vitro*, depending on the assay, than LPS from *Enterobacteriaceae*. Our results for *Bacteroides* LPS extracted by the PCP or Triton methods agree with this. However, our results for LPS extracted by the PW method disagree with the accepted view. In the TNF induction assay, *E. coli* O18K$^-$ LPS was only 4-fold more active than *B. fragilis* NCTC 9343 LPS and 7-fold more than *B. caccae* MPRL 1555 LPS but up to 50-fold more than the other *Bacteroides* LPSs.

In the LAL assay, (a standard *in vitro* method for detection of endotoxin), which has questionable value to the situation *in vivo*, the same LPSs which were most active in

the TNF-induction assay (from *B. fragilis* and *B. caccae*) were 30-40 fold more active than *E. coli* O18K$^-$ LPS, with *E. coli* O18K$^-$ LPS being approximately 10-fold more active than the rest of the *Bacteroides* LPS tested. In a study by Laude-Sharp *et al* (1990) no relationship between IL-1 inducing capacity and LAL reactivity was found for various LPSs from *Enterobacteriaccae*. They describe how biologically active LPS species can escape recognition in the LAL assay which only detects LPS of molecular mass above 8,000 Da.

The biological activity of enterobacterial LPS is thought to be dependent on its solubility (Galanos & Luderitz 1984), and for this reason the native and soluble sodium salt forms were prepared from electrodialysed material. Results found that solubility (native *vs.* sodium salt form LPS) does not play an important role in biological activity as has been previously described for enterobacterial LPS (Galanos & Luderitz 1984). In most cases the sodium salt form was of lower activity.

It must be stressed at this stage that the term "*Bacteroides* LPS" is probably not a single molecular species, of even heterogeneous chain length, but is much more likely to be a complex of surface lipopolysaccharides, both rough and smooth, as well as possibly lipid-linked CP. In SDS-PAGE the *Bacteroides* LPS appears as a series of several different molecules of high and low molecular mass (Fig 6). To add to the complexity, some of the molecules can only be detected by immunostaining as they are not sensitive to staining by silver (Allan *et al* 1995). From the work of Patrick and colleagues (Patrick 1993, Patrick & Reid 1983) we know that a pure laboratory culture of *B. fragilis* exists as a collection of sub-populations expressing a range of surface antigens. These sub-populations can be separated on Percoll density gradients, and the different antigens identified with a series of mAbs (Lutton *et al* 1991). The degree of different molecular species of LPS and other co-purifying carbohydrate, due

to the probable sub-populations of cultures in this study, is by no means certain. Because of this complexity of the different molecular species, the comparison of activity with *E. coli* O18K⁻ LPS on a weight-for-weight basis is preferred to a comparison on a molar basis.

4.2 DIFFERENCES IN BIOLOGICAL ACTIVITY BETWEEN *BACTEROIDES* SPECIES

Some PW preparations of *Bacteroides* LPSs are clearly more active than others. For example in the LAL, TNF, IL-8 and complement CH_{50} assays *B. fragilis* NCTC 9343 VT/PPY and *B. caccae* MPRL 1555 were consistently the most active *Bacteroides* LPSs. In the same assays, *B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720 were consistently the least active LPSs. However, in the mitogenicity assays, *B. vulgatus* MPRL 1985 LPS was more active than *B. fragilis* NCTC 9343 LPS (see section 4.8 for further discussion).

Growth in the defined VT medium was included because a recent study in our laboratory found that growth in this medium converts several *B. fragilis* strains, including NCTC 9343, from being sensitive to the lytic action of normal human serum to resistant. This may be due to a change in expression of certain surface polymers such as LPS (Allan & Poxton 1994). Results found in this study show that LPS from *B. fragilis* NCTC 9343 grown in VT is more active in the LAL and TNF assays and less active in the mouse lethality model compared to *B. fragilis* NCTC 9343 LPS grown in PPY.

Due to anaerobic conditions in the gut, *E. coli* O18K⁻ LPS was grown anaerobically as well as aerobically. There was no difference in activity between *E. coli* O18K⁻ LPS grown aerobically and anaerobically apart from in the LAL assay. In this assay, the

two-fold difference in activity for *E. coli* O18K⁻ LPS grown aerobically and anaerobically is unclear.

4.3 REASONS FOR DIFFERENCES IN BIOLOGICAL ACTIVITY BETWEEN EXTRACTION METHODS AND *BACTEROIDES* SPECIES

Chemical analysis does not explain clearly why the LPS from some species are more active than others. It does show that more carbohydrate and KDO is present in the PW preparations. The amount of KDO destroyed or not released by hydrofluoric acid treatment is not known. *B. fragilis* NCTC 9343 VT LPS had more carbohydrate and KDO than *B. fragilis* NCTC 9343 PPY LPS. Fatty acid analysis was not conclusive and its reliability debatable when we consider that results found do not relate to those previously accepted. Lindberg and colleagues (1990), found that C14, the most common enterobacterial fatty acid was absent in *Bacteroides*. However, in this study (see Table 5) a small quantity of this fatty acid was found in *B. fragilis* NCTC 9343 PPY PW-LPS. It has previously been found that *B. fragilis* LPS contains fatty acids C15-C17 and *E. coli* LPS C12-C16, but in this study fatty acids C12-C13 were present in *B. fragilis* NCTC 9343, *B. thetaiotaomicron* MPRL 1720 and *B. vulgatus* MPRL 1985 LPS. Differences in detected fatty acids may be due to different sub-populations present in cultures used and the extraction method employed. Clearly, further analysis is required to clarify results.

Those species that had a low reactivity in the LAL assay, TNF and IL-8 bioassays, and mouse lethality model (*B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720) had a smooth LPS profile on PAGE for all extraction methods (Fig 6). This is in apparent contradiction to the view that aqueous phenol preferentially extracts smooth-form LPS of higher activity, while those strains which are obviously smooth (e.g. *B. vulgatus*) are less endotoxic. However, even the highest molecular band is

much lower than the highest enterobacterial LPS bands. Background information into sub-populations, Percoll profiles and Proteinase K profiles failed to account for any differences in activity between *Bacteroides* species as well.

The possibility that some contaminant may be present in the LPS preparations from *Bacteroides* must be considered. In this connection, no contaminating organisms were found when culturing the organisms for extraction and the identity of each strain of *Bacteroides* was confirmed prior to the start of the study. Furthermore, PW-LPS samples from several *Bacteroides* species prepared by different individuals at different times in our laboratory were tested alongside our own samples in the LAL and TNF induction assay. They were found to produce near identical results (data not shown). Higher biological activity in the PW preparations cannot be due to a biologically active protein as protein levels were negligible after purification. LPS samples with protein contamination had lower activity in the TNF and LAL assays (Tables 8 and 10). Biological activity also cannot be due to any contaminating Proteinase K as all *Bacteroides* LPSs had been treated similarly with Proteinase K.

4.4 INDUCTION OF CYTOKINES BY *BACTEROIDES* LPS

4.4.1 General Comments

Several points must be kept in mind when drawing conclusions from this area of work. As mycoplasma screening had not been carried out on tissue culture cells, the effect of contaminating mycoplasma cannot be ruled out. Cell lines were not thought to be contaminated as retrospective testing showed no presence of mycoplasmas (Ciara Ryan, personal communication).

Choice of LPS level

As previously discussed in section 3.10.5, the amount of LPS used to stimulate secretion of cytokines was chosen as it fell in the middle of the linear part of the standard curve of the assay employed in this thesis. This amount of LPS (50 ng/ml) may be felt to be particularly high compared to the situation *in vivo* in the bloodstream, but this amount of LPS may resemble the situation in abscesses and other sites of infection where the local concentration of LPS may be higher. Circulating levels of LPS *in vivo* are measured by the LAL assay and represent "excess" LPS. LPS bound or masked by serum proteins may not trigger the LAL assay and therefore circulating levels of endotoxin may be higher than measured.

LPS is only thought to interact with CD14/LBP when circulating levels of LPS are low (< 1 ng/ml) and the level of LPS employed during the functional studies may be deemed too high to refer any conclusions from the interactions of LPS with CD14. Since levels of 1 ng/ml of LPS only gave baseline levels of cytokines in the assay systems employed in this thesis, a larger quantity (i.e. 50 ng/ml) of LPS was used. Clearly the situation *in vivo* cannot be reproduced *in vitro*.

Choice of CD14 mAb

The binding characteristics of the CD14 mAb employed in this thesis are not known. It was chosen due to its abundant, free availability. At this stage it was deemed important to find out if neutralisation occurred. If it did, then the study could be extended to employ a mAb of known binding site.

Sodium azide is commonly found in many commercial mAb preparations. The presence of sodium azide may kill tissue culture cells and therefore affect results in the assay systems employed in this thesis. The presence or absence of sodium azide in

the CD14 preparation used in this thesis cannot be confirmed. If the killing of L929 cells was due to sodium azide alone we would not have seen a differential effect as was seen with the different LPSs. These studies would have benefited from a matched isotype mAb control. Antibody preparation available in our laboratory had significant endotoxin levels present as determined by the LAL assay and the best control was therefore thought to be no antibody.

4.4.2 Induction of Tumour Necrosis Factor

Bacteroides LPS is capable of inducing mononuclear cells to produce TNF, which is considered the pivotal cytokine in the host response to endotoxin. This induction seems to be independent of CD14 expression, the 55 kD glycosyl phosphatidylinositol-linked protein expressed on the surface of monocytes, macrophages and PMNs (Haziot *et al* 1988), which has been found to serve as a specific binding site for complexes of LPS and LBP in serum (Lynn & Golenbock 1992, Wright 1991, Wright *et al* 1990). Although CD14 has been shown to play a very important role in LPS activation events, its functional role has yet to be fully defined.

It is well documented that immature THP-1 cells are lacking in CD14 and do not respond as well to LPS as THP-1 cells that have been matured with vitamin D3 (Martin *et al* 1994, Kitchens *et al* 1992). Flow cytometric analysis determined that THP-1 cells without enhancement for CD14 were lacking in CD14 (Table 24). In this cell population, *E. coli* O18K$^-$ LPS could only induce one peak of TNF release at 4 h, compared to peaks at 4 h and 8 h in CD14-rich populations (human MNL and THP-1 cells with enhancement for CD14 (Table 24, Figs 10 and 11). The peak of TNF release at 8 h was inhibited by high concentrations of the anti-CD14 mAb (Fig 15). *Bacteroides* LPS could only induce one peak of TNF release at 4 h regardless of the

cell population, suggesting that it stimulates cells to produce TNF via a pathway independent of CD14 and that the periodic response may be related to CD14. The fact that TNF release stimulated by *Bacteroides* LPS could not be inhibited by an anti-CD14 mAb (compared to *E. coli* O18K$^-$ LPS which could be inhibited), is further evidence to support this concept. These results can be contrasted to those found in a study by Shapira *et al* (1994), where TNF production by human MNL by *E. coli* LPS and *Porphyromonas gingivalis* LPS (once a member of the *Bacteroides* genus) was inhibited by an anti-CD14 mAb. Of note is that only in human cell populations rich in CD14 is there a significant difference in TNF inducing capability by LPS from *Bacteroides* and *E. coli* O18K$^-$.

The presence or absence of serum does not effect TNF production by *Bacteroides* LPS, whereas the absence of serum greatly reduces the ability of *E. coli* O18K$^-$ LPS to induce TNF (Table 11). This suggests that *Bacteroides* LPS does not require the presence of serum proteins to activate cells to produce TNF, and that *E. coli* O18K$^-$ LPS can activate cells without the presence of serum proteins which is greatly enhanced by the presence of serum proteins. Serum proteins derived from both FCS and human serum produce the same effects (Table 11), suggesting a degree of homology between the species. It is not known what amount of LBP, if any, is present in the serum used. The addition of LBP to the culture medium could not be carried out due to its lack of commercial availability and to its complex purification from serum.

In a recent paper by Lynn *et al* (1993) neither CD14 or serum were shown to be absolutely necessary for the activation of mononuclear phagocytes by bacterial LPS. They suggested that a CD14-independent pathway may be of importance in local sites of infection where the concentration of LPS may be high: for example, faecal soiling

of the peritoneum. Local concentrations of LPS may be high in abscesses which may be reason why *Bacteroides* has evolved to trigger cells to produce cytokines without the need for CD14. Peritoneal macrophages may differ in their ability to respond to LPS compared to circulating macrophages. It has been reported that different macrophage populations differ in their TNF response to LPS: alveolar macrophages for example, produce a much higher level of TNF with a faster response than Kuppfer cells (Callery *et al* 1991). It must be remembered that the peritoneal macrophage preparation used in this thesis contained contaminating neutrophils and lymphocytes. It is not known at this stage what amount of cytokines is produced by each of these cell types.

In peritoneal macrophages from C3H/HeJ mice the *B. fragilis* NCTC 9343 LPS stimulates a low level of TNF whereas *E. coli* O18K$^-$ LPS stimulates no TNF production. It is well documented that peritoneal macrophages from C3H/HeJ mice are unable to respond to LPS from *Enterobacteriaceae* due to a genetic deficiency (Sultzer 1976). However, a pathway open to *B. fragilis* LPS is capable of stimulating TNF production. The component of *B. fragilis* NCTC 9343 LPS complex which is inducing this effect and through which pathway is not yet known. Other *Bacteroides* species have yet to be investigated.

It appears that the amount of TNF produced is also directly related to the experimental volume. When the experimental volume was small, cells had a greater tendency to pellet out on the bottom of the microtitre plates. This suggests that the LPS has a greater probability to interact with cell surface receptors, which could explain greater amounts of TNF produced. This effect has also been observed by other workers (J. Pitts, Personal Communication).

4.4.3 Induction of Interleukin-8

IL-8 is a powerful chemoattractant for PMNs, recruiting them to a site of infection such as intra-abdominal abscesses (Janeway & Travers 1994, Neilson *et al* 1994). Abscesses consist primarily of PMNs, some macrophages, bacteria and cellular debris. Since *B. fragilis* is the predominant organism found in such abscesses, it was logical to determine the ability of *B. fragilis* LPS to induce this cytokine.

In contrast to the production of TNF by human MNL, where *E. coli* O18K$^-$ LPS induced a periodical TNF response with major peaks at 4 and 8 h and *B. fragilis* NCTC 9343 LPS induced one peak (if somewhat complex-shaped) of TNF at 4 h, the kinetics of IL-8 induction by these LPSs was different. In this cell population, *E. coli* O18K$^-$ LPS induced a single peak of IL-8 at 6 h and *B. fragilis* NCTC 9343 LPS induced three peaks of IL-8 at 6, 9 and 12 h (Fig 18).

Similarly the kinetics of IL-8 production from THP-1 cells with and without enhancement for CD14 differed from those found for TNF production. In THP-1 without enhancement for CD14, *E. coli* O18K$^-$ LPS induced a single peak of IL-8 at 4 h and *B. fragilis* NCTC 9343 LPS induced two peaks of IL-8 at 4 and 10 h. The amount of IL-8 produced in THP-1 cells with enhancement for CD14 was much larger and produced a single peak of IL-8 at 8 h by *E. coli* O18K$^-$ LPS and two peaks at 4 and 8 h by *B. fragilis* NCTC 9343 LPS (Fig 18). Of note is the fact that *B. vulgatus* MPRL 1985 LPS induced more IL-8 than *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 LPS. However, since these results are the means of duplicates from a single experiment, their reliability could be debated.

The kinetics of IL-8 production found in this study differ from those previously described (DeForge *et al* 1992). In that study a single peak of TNF/IL-6 and a

second peak of both mRNA and protein IL-8 was described following stimulation with *E. coli*. It is not known if this effect is due to stimulation in whole blood rather than purified MNL as in this study, or to the type of LPS preparation used.

The production of IL-8 by both *E. coli* O18K$^-$ LPS and *B. fragilis* NCTC 9343 LPS could be slightly inhibited by an anti-CD14 mAb (Fig 20). The amount of inhibition is not significant enough to conclude that both these LPSs activate cells to produce IL-8 through a pathway involving CD14. Although IL-8 production is greatly increased in THP-1 cells which have been enhanced for CD14, it is not known at this stage if the slight inhibition seen is due to the presence of antibody in the assay system. This is where the presence of a matched isotype mAb control would have been beneficial. The fact that *B. fragilis* NCTC 9343 LPS can induce multiple peaks of IL-8 in all cell populations tested may have some significance in the abscess model.

4.4.4 Induction of Interleukin-6

One of the most important multi-functional activities of IL-6 is the initiation of the acute phase response. IL-6 acts on hepatocytes in the liver to induce the production of acute phase proteins such as C-reactive protein.

In all cell populations tested, both *E. coli* O18K$^-$ LPS and *B. fragilis* NCTC 9343 LPS induced a single comparable peak of IL-6 at between 6-8 h (Fig 23). The production of IL-6 by both LPSs could not be inhibited by an anti-CD14 mAb (Fig 25) suggesting that they induce cells to produce IL-6 through a pathway independent of CD14. Further evidence to support this concept is the fact that the amount of IL-6 produced by both LPSs is nearly identical in THP-1 cells with and without enhancement for CD14.

Clearly from the results found in this study, TNF at 4 h is the first cytokine to be induced from all cell populations tested by both *E. coli* O18K⁻ LPS and *Bacteroides* LPS. The production of IL-6 and IL-8 follows a little while later at between 6-8 h. The influence of TNF in the production of IL-6 and IL-8 in the cell models used in this study is not known at this stage, but has been reported to influence their production by many other workers (DeForge *et al* 1992, Matsushima & Oppenheim 1989, Janeway & Travers 1994). Why both *E. coli* O18K⁻ LPS and *B. fragilis* NCTC 9343 LPS should utilise CD14 to induce some cytokines and not others is not known. This phenomenon has been observed by other workers. In a study by Le Dur *et al* (1994), they observed that CD14 induced the signal for TNF but not IL-1 from human MNL stimulated with LPS. The activation pathway independent of CD14 is not known at present.

The ability of LPS to induce TNF and interact with CD14 has been reported to be connected with the chain length of the LPS (Jahr *et al* 1994), where the longer the chain length of the LPS, the more able it is to interact with CD14. If *Bacteroides* LPS is more of a rough form than *E. coli* O18K⁻ LPS then this may explain results found for TNF induction assays. However, this theory would contradict those results found for IL-8 and IL-6 assays.

4.5 INDUCTION OF CYTOKINES BY CAPSULAR POLYSACCHARIDES FROM *B. FRAGILIS* NCTC 9343

The CP of *B. fragilis* has been seen to consist of at least two different molecules (Patrick 1993, Tzianabos *et al* 1993, Tzianabos *et al* 1992). An intriguing observation was made several years ago by Onderdonk and colleagues (Onderdonk *et al* 1977) that the CP of *B. fragilis* could induce sterile abscesses. This has recently been re-addressed (Tzianabos *et al* 1993), and Cross (Cross 1994) has suggested that CP may

induce proinflammatory mediators such as IL-1. It has previously been reported that some preparations of CP are contaminated with high molecular mass LPS (Poxton & Brown 1986) and as mentioned before in section 4.1, *Bacteroides* LPS preparations used in this study may not be pure but heterogeneous with lipid-linked CP. For this reason, the ability of CP from *B. fragilis* NCTC 9343 to induce cytokines was examined.

Due to the very small quantity of CP prepared, a fully comprehensive study was not possible. Although purification of polysaccharide A and B was attempted, not enough material to carry out extensive studies with these fractions was obtained. Most cytokine-induction capacity was seen in the low molecular weight CP fraction. This fraction contained KDO whereas the high molecular weight CP fraction contained no KDO. The low mwt CP fraction is most likely to be pure LPS and the high mwt CP is most likely to be pure CP. Both the low and high molecular weight CP induced TNF from human MNL on a scale much less than *B. fragilis* NCTC 9343 LPS. Both induced a single peak of TNF like *Bacteroides* LPSs but later at 6 h (Fig 17). The low mwt CP induced a single peak of IL-8 at 10 h and in contrast the high mwt CP induced increasing levels of IL-8 with a maximal level at 24 h comparable to the levels seen for the LPSs (Fig 21). The significance of the amount of IL-8 produced by the high mwt CP in abscess formation is not known at this stage. The kinetics of IL-8 production following stimulation with high mwt CP resembles that by *E. coli* in a study by DeForge *et al* 1992. The role of CD14 in CP cell activation is also not known at this stage. Further biological activities of CP are discussed in sections 4.6 and 4.8.

4.6 EFFECT ON CYTOKINE INDUCTION BY *E. COLI* O18K⁻ LPS WHEN *B. FRAGILIS* NCTC 9343 LPS OR CAPSULAR POLYSACCHARIDE PRESENT IN EXCESS

An excess of *Bacteroides* LPS (by all three extraction methods) or CP (particularly low molecular weight CP) blocked the effects of *E. coli* O18K⁻ LPS on human MNL and THP-1 cells (with and without enhancement for CD14) in producing the cytokines TNF, IL-8 and IL-6 (Figs 12, 19, 24 and Table 19). This effect was also seen in the complement CH_{50} assay (Table 27). In TNF induction, an excess of *B. fragilis* NCTC 9343 LPS inhibited the second peak of TNF production at 8 h previously seen by *E. coli* O18K⁻ LPS (Fig 13). Most inhibition was seen when both LPSs were added to cell populations at the same time (Table 14). Whether this was purely due to the configuration of LPS in solution or due to an effect at the cellular level is not known.

A similar phenomenon has been reported by Ogawa *et al* (1994a) where they report that the production of IL-1β by *E. coli* is inhibited by *Porphyromonas gingivalis* (once a member of the *B. fragilis* group) LPS and its lipid A. Their results also mimic those found in this study where the difference in IL-6 and IL-8 production by both *E. coli* and *P. gingivalis* LPS was less marked.

A well documented synergistic relationship exists between *E. coli* and *B. fragilis*, where *B. fragilis* inhibits the phagocytic killing of *E. coli* (Onderdonk *et al* 1976, Rotstein *et al* 1989). Magnuson *et al* (1989) also found that *B. fragilis* NCTC 9343 LPS inhibits *E. coli* LPS-induced human endothelial cell adhesiveness for neutrophils. However, in that study it was not found that *B. fragilis* LPS inhibited TNF production, but the *B. fragilis* LPS was extracted sequentially by the phenol-water method followed by the phenol-chloroform-petroleum method. This contradicts

findings in this study where *B. fragilis* NCTC 9343 LPS by all three extraction methods inhibited TNF production by *E. coli* O18K⁻ LPS.

These observations in this study may be of some importance when we consider that *Bacteroides* outnumber *E. coli* in the gut by at least 1000-fold. It is not yet known if the situation observed *in vitro* will occur *in vivo*. However, it may be significant in terms of immunotherapy if bacteria or their products translocate from the gut into the bloodstream. If *Bacteroides* LPS is the major inducer of cytokine responses we should be targeting them with antibody therapy instead of *E. coli* (Baumgartner 1994). However, if the inhibition of the *E. coli* LPS response occurs *in vivo* then perhaps the *Bacteroides* LPS might serve a protective role.

4.7 INHIBITION OF CYTOKINE PRODUCTION BY ANTI-*BACTEROIDES* MONOCLONAL ANTIBODIES

Some anti-*Bacteroides* mAbs could inhibit TNF production from human MNL (Table 15) but not IL-8 production (Table 21). Although these mAbs were raised against *B. fragilis* NCTC 9343 they displayed a degree of cross-reactivity. At this stage it is not known to which epitopes they are cross-reactive. The mAbs 5A12, 1A4 and 3D7 were cross-reactive with *E. coli* O18K⁻ LPS and *B. fragilis* NCTC 9343 LPS. The mAbs 5A12, 3D7 and 3C8 cross-reacted slightly with *B. caccae* MPRL 1555. The mAb 6g3 and 4CS had little or no activity in inhibiting TNF production and did not bind in ELISA suggesting that little or no antibody was present in the culture supernates. The mAb 5A12, which was against the complete LPS ladder pattern on PAGE was the most successful in inhibiting TNF production. The mAbs 1A4 and 3D7 were the next most successful in inhibiting TNF production. These mAbs were against the high molecular material on PAGE, however, other mAbs to the high molecular material such as 4CS and 3C8 were less successful. In general the binding

of these mAbs to various LPSs in ELISA was poor due to the probable low concentration of mAbs in the culture supernates (Table 28). Interestingly, 5A12 bound most strongly to *E. coli* O18K$^-$ LPS in ELISA, with the rest of the mAbs binding most strongly to *B. fragilis* NCTC 9343 LPS. The mAbs 3D7 and 6G3 showed no binding in ELISA.

As discussed in section 4.5 most TNF-induction capacity lies in the low molecular weight material and not the high molecular weight material. How mAbs directed against the high molecular weight material can therefore prevent TNF production is unclear. This may be due to the configuration of LPS in solution. It is not known if the reduction of TNF production due to the action of the anti-*Bacteroides* mAbs would in turn reduce the amount of other cytokines produced such as IL-6 or IL-1. Of note is the fact that when *B. fragilis* NCTC 9343 LPS is present in excess of *E. coli* O18K$^-$ LPS together with the mAb 5A12 (Fig 16), the re-emergence of TNF levels by *E. coli* O18K$^-$ LPS alone is not seen, but the amount of TNF produced is further reduced. If *Bacteroides* LPS were to prove as an important factor in the development of sepsis then a mAb against the whole LPS ladder for use in immunotherapy such as 5A12 would be preferred.

4.8 MITOGENICITY OF *BACTEROIDES* LPSs AND CAPSULAR POLYSACCHARIDES

LPS has been well described as a potent activator of B cells (Chaby & Girard 1993). B cells can bind LPS without macrophage activation and can also be activated by cytokines released from mononuclear cells stimulated by LPS. Some authors have reported binding of LPS to T cells, others concluded that LPS cannot bind to T cells (Haeffner-Cavaillon *et al* 1985).

It was first recognised by Joiner and colleagues in 1982 that LPS from *B. fragilis* 23745 showed potent mitogenic activity for spleen cells from both responder and non-responder mice. Williamson *et al* (1984) corroborated these findings and recognised that the polysaccharide and lipid A fractions of *B. fragilis* LPS (prepared by aqueous phenol extraction) had different effects on macrophages of C3H/HeJ (non-responder) mice. The mitogenic response was only to the polysaccharide fraction. Fujiwara *et al* (1988) further demonstrated that LPS extracted by aqueous phenol from oral *Bacteroides* species (now reclassified as *Porphyromonas* and *Prevotella*) were highly mitogenic for peritoneal macrophages from C3H/HeJ mice. LPSs from *Enterobacteriacae* are not mitogenic for C3H/HeJ mice. However when the LPS is extracted with hot trichloroacetic acid or aqueous butanol, it contains a lipid A associated protein which is mitogenic for both LPS responder and LPS non-responder mouse spleen cells (Morrison 1976).

It has also been observed that T cells (phenotypically T helper or precursor cells) are required for the induction of abscesses caused by *B. fragilis.* Protection against abscess formation has been found to be a consequence of suppressor T cell activity (Shapiro *et al* 1986). No literature on mitogenic responses for *E. coli* LPS to T cells has been found.

Studies on mitogenicity of LPS in this thesis were not successful. Flow cytometric analysis could not demonstrate that homogeneous cell populations were obtained. Fluorescence was seen on macrophage populations and there was no difference in fluorescence between those cells treated with and without an anti-CD3 mAb (Table 25). This suggests non-specific binding of the FITC-labelled mAb. Attempts to repeat these experiments using a different FITC-labelled mAb were made, but due to

repeated failure of the T cell columns, this was not possible. Because of these problems, these studies were not extended to non-responder mice.

Although firm conclusions from the mitogenic studies cannot be made, several observations are of note. Generally, the high mwt CP was more active than the low mwt CP. This ties in with findings previously described by Williamson *et al* (1984) who found that only the polysaccharide fraction was mitogenic. LPS from *B. vulgatus* MPRL 1985 was more active than *B. fragilis* NCTC 9343 LPS in all cell populations apart from in the T cell population where *B. fragilis* NCTC 9343 LPS was more active (Tables 22 and 23). This is in contrast to results found in the cytokine assays where LPS from *B. vulgatus* MPRL 1985 was consistently of lower activity. *B. vulgatus* LPS appears smooth on PAGE (Fig 6) which could indicate that more polysaccharide is present. The fact that a mitogenic response was seen in the T cell population for all samples is further evidence to suggest that homogeneous cell populations were not achieved.

It has been described that inhibition of LPS internalisation by neuraminadase treatment prevents mitogenic responses of B cells to LPS (Chaby & Girard 1993). *B. fragilis* is known to secrete neuraminadases (Imai *et al* 1991). It is not known at this stage whether viable *B. fragilis* bacteria would activate B cells in comparison to LPS and CP *in vivo*.

4.9 UNANSWERED QUESTIONS FROM THIS STUDY

There are still many questions that remain to be answered.

i) Since chemical analysis in this study has been non-conclusive, what are the reasons behind the differences in biological activity between *Bacteroides* species? What type of extracted LPS mimics that *in vivo*?

ii) Why are there several peaks of TNF production after stimulation with *E. coli* LPS in CD14-rich cell populations? What causes the "troughs" in the periodic TNF response? It is acknowledged that there may be heterogeneity in the cell population or different stages present in the cell cycle which may cause this response. Also TNF may be present during the "trough" but in a bound form which would not be detectable in the bioassay. The possibility that TNF is neutralised during the "trough" by the p55 sTNFr appears unlikely (Fig 26). The role of the p75 sTNFr (which has a greater affinity for TNF) in this regard is unknown at this stage.

iii) What is the activation pathway which is independent of CD14 in the production of TNF by *Bacteroides* LPS and by both *E. coli* O18K$^-$ LPS and *Bacteroides* LPS in the production of IL-6, and how does *Bacteroides* LPS stimulate peritoneal macrophages from C3H/HeJ mice to produce TNF?

iv) Does *Bacteroides* LPS utilise CD14 in the production of IL-8, and does *Bacteroides* LPS bind to LBP? If *Bacteroides* LPS does bind to LBP *in vivo*, could its lower biological activity be due to its preferential neutralisation by HDL? Studies should be extended to include a CD14 mAb of known binding site to assist in finding these answers.

v) How does an excess of *Bacteroides* LPS mask the effects of *E. coli* LPS? Does this effect observed *in vitro* occur *in vivo*? Is it the physical state of the LPS mixture or is there competition or masking of binding sites?

vi) What species of LPS is circulating in the septic patient and in what form is it predominant?

vii) Neutrophils are a characteristic of intra-abdominal abscesses and are the most common leukocyte in the bloodstream. Since *B. fragilis* is the predominant organism in intra-abdominal abscesses and are capable of inducing IL-8 (a powerful chemoattractant for neutrophils), what role does the neutrophil play in *Bacteroides* LPS-induced cytokine response?

viii) Only a fraction of the cytokines produced *in vivo* have been examined, does *Bacteroides* LPS induce other cytokines in a significant amount compared to *E. coli* LPS ?

There is still therefore much to be learnt about *Bacteroides* LPS monocyte/macrophage activation pathways and its role in the cytokine cascade.

4.10 CONCLUSIONS

Despite many unanswered questions, many points can be concluded from this study.

i) PW extracted *Bacteroides* LPS is more active than PCP or Triton extracted LPS.

ii) *B. fragilis* NCTC 9343 and *B. caccae* MPRL 1555 LPSs are consistently the most active *Bacteroides* LPSs, with *B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720 LPSs consistently the least active LPSs.

iii) In TNF production, *E. coli* O18K$^-$ LPS can produce multiple peaks through a CD14-dependent pathway while *B. fragilis* NCTC 9343 LPS can only produce one peak through a pathway independent of CD14. Both low and high mwt CPs induce one peak of TNF. Low mwt CP induces more TNF than high mwt CP but on a scale much lower than both *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 LPS.

iv) In IL-8 production, *E. coli* O18K$^-$ LPS can produce one peak while *B. fragilis* NCTC 9343 LPS can produce two peaks. Low mwt CP induced one peak of IL-8 whereas high mwt CP induces an increasing level of IL-8 over 24 h.

v) In IL-6 production, both *E. coli* O18K$^-$ LPS and *B. fragilis* NCTC 9343 LPS produce one peak through a pathway independent of CD14.

vi) An excess of *B. fragilis* NCTC 9343 LPS and CP can mask the effects of *E. coli* O18K$^-$ LPS.

vii) Firm conclusions from the mitogenic studies cannot be drawn, but a trend towards high mwt CP being more mitogenic than low mwt CP and *B. vulgatus* MPRL 1985 LPS being more mitogenic than *B. fragilis* NCTC 9343 LPS was seen.

B. fragilis has a history of debate and some confusion as to the structure/function relationships of its polysaccharide surface structures (Lindberg *et al* 1990, Poxton & Brown 1986). It is perhaps too early at this stage to suggest that individual molecules

can induce specific mediators. However, until we can finally fractionate, or clone, the various other constituent molecules, little further progress can be made in determining their individual and combined functions in pathogenesis. The role of *Bacteroides* LPS in shock remains to be defined, but by taking the results of this study, *Bacteroides* LPS may play an important but neglected role in endotoxic shock.

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A re-appraisal of the biological activity of bacteroides LPS

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Summary. Lipopolysaccharides (LPS) were extracted from seven *Bacteroides* strains by three different techniques: the phenol-water (PW), phenol-chloroform-petroleum (PCP) and Triton-Mg^{2+} methods. The strains selected included two different *B. fragilis* strains, one of which was grown in two different media. Yields varied between the strains, growth media and extraction technique, but generally the highest yield by weight was from the PCP method and the lowest from the PW method. The PW method was selected for the greatest amounts of carbohydrate and KDO, and the PCP method for the least. Phosphorus levels were more uniform among all extraction methods. Protein contamination was found in all *Bacteroides* LPS extracts, with extremely low levels in PW-LPS and the highest levels in material extracted by the PCP and Triton-Mg^{2+} techniques. No protein contamination could be detected after proteinase K treatment. After silver staining LPS PAGE profiles showed ladder patterns characteristic of smooth LPS for *B. vulgatus*, *B. thetaiotaomicron* and the control *Escherichia coli* O18:K^- strains, whereas the other *Bacteroides* strains showed mainly rough and low M_r material only. The PCP method did not select for high M_r material in the *B. fragilis* strains; otherwise the LPS profiles for all extraction methods were identical. The biological activities of native and sodium salt form LPS were investigated on a weight for weight basis and compared to that of *E. coli* O18:K^- PW-LPS. Amongst the LPS from *Bacteroides* strains, those prepared by the PW method were found to have a significantly higher activity in a galactosamine mouse lethality model, in induction of TNF and the *Limulus* amoebocyte lysate (LAL) assay, than LPS extracted by the PCP or Triton-Mg^{2+} methods. LPS from *Bacteroides* strains extracted by the PCP method had consistently low activity in all assays. Comparing PW-LPS from *Bacteroides* strains with that from *E. coli* O18:K^- in the galactosamine mouse model, the *E. coli* O18:K^- LPS was *c.* 5000-fold more active than the most active bacteroides LPS. However, in the LAL assay native PW-LPS from both the *B. fragilis* strains, and *B. caccae* had higher activities (up to 30-fold) than *E. coli* O18:K^- LPS, with the PW-LPS from the other *Bacteroides* spp. being up to 15-fold less active than the *E. coli* O18:K^- PW-LPS. In the TNF induction assay, *E. coli* O18:K^- PW-LPS was 4–50-fold more active than bacteroides PW-LPS. In the LAL assay and galactosamine mouse model, native LPS had more activity (*c.* two-fold) than sodium salt form LPS. There was no clear difference in activity between native and sodium salt form LPS in the TNF induction assay. The results for the LAL and TNF induction assay were re-evaluated relative to KDO concentration. In the TNF induction assay, previously low activities seen on a weight for weight basis were due in part to less KDO being present. However, LAL activity for PCP-LPS was still low after re-evaluation relative to KDO concentration. The molecular basis for the differences in biological activity of bacteroides LPS in relation to extraction methods and chemical composition is not yet understood.

Introduction

The gram-negative bacillus *Bacteroides fragilis* is the anaerobe most frequently isolated from human infections including intra-abdominal, vaginal, brain and lung abscesses and peritonitis. The source is usually the faecal flora. Faeces contain 10^{11}–10^{12} bacteria/g and anaerobes constitute *c.* 99·9% of the faecal bacterial mass. Of this, the species belonging to the genus *Bacteroides sensu stricto* (formerly members of the *B. fragilis* group of *Bacteroides*) account for 20–30% of

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the species isolated.[1] The genus *Bacteroides* constitutes *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*, with *B. eggerthii*, *B. uniformis*, *B. caccae*, *B. merdae* and *B. stercoris* being more recently added to the group due to changes in classification. Other species formerly classified as *Bacteroides* have been reclassified into the genera *Porphyromonas* and *Prevotella*.[2,3]

In the past 25 years, the lipopolysaccharide (LPS) of *B. fragilis* has been studied by many groups and has yielded many controversial results regarding its rough or smooth structure, and presence of KDO or not, but it is generally agreed that it is weakly endotoxic.[4–7] It was thought initially that *B. fragilis* LPS contained no repeating long chains of polysaccharide linked to lipid A core. This was due in part to the use of the phenol-chloroform-petroleum extraction method which tends to select for rough type LPS[5] and it was not recognised that subpopulations existed that expressed different surface molecules.[3] In 1991[8] Maskell investigated by polyacrylamide gel electrophoresis LPS from 10 species of *Bacteroides* prepared by the non-selective proteinase K method. He showed that some *Bacteroides* spp. produced predominantly rough LPS whereas others such as *B. vulgatus* and *B. caccae* produced smooth LPS similar to that of facultative enterobacteria. Other species such as *B. fragilis* produced poorly resolved high molecular mass (M_r) material as well as rough LPS. It is now well established that *B. fragilis* LPS does contain KDO, but only in a phosphorylated form which renders it undetectable in the standard thiobarbituric acid assay. Prior dephosphorylation by hydrofluoric acid treatment is required for its detection.[9] It is well documented that the lipid A of *B. fragilis* LPS contains different fatty acids from the lipid A of enterobacterial LPS and is monophosphorylated, and this is one reason why it is less endotoxic than enterobacterial LPS.[7]

Endotoxaemia can occur as a primary event, for example in patients with intra-abdominal or urinary tract sepsis. However, in critically ill patients without a confirmed bacteraemia, it is thought that the gut is the major source of "endotoxin"[10] which subsequently leads to the systemic inflammatory response syndrome (SIRS),[11] a consequence of which can be multiple organ failure and death in up to 80% of patients. Although it has been shown that the LPS of *B. fragilis* has lower activity than enterobacterial endotoxin,[7] *Bacteroides* spp. are numerically far more predominant (*c.* 1000-fold) in the gut than enterobacteria such as *E. coli*, thus representing a vast pool of potentially biologically active LPS.

In this paper, the biological activity of LPS from seven species of *Bacteroides* is re-examined by comparing the activity of LPS extracted by three different methods in mouse lethality and TNF induction models, and an attempt is made to relate activity to chemical composition. The *Limulus* amoebocyte lysate (LAL) assay was included in the study as the standard in-vitro method for detection of endotoxin.

Materials and methods

Bacterial strains

A list of strains used and their origin is shown in table I.

Culture methods

Bacteria were grown in 15–16-L batches in a 20-L capacity fermenter (LH Engineering, Stoke Poges). *Bacteroides* spp. were grown in defined broth[12] or proteose-peptone yeast extract (PPY) broth, anaerobically, in N_2 100% at 37°C. *Escherichia coli* O18:K$^-$ was grown in nutrient broth (NB) anaerobically, in N_2 100% at 37°C, or aerobically (pO_2 50%) at 37°C. Strict purity checks were made for each organism at the end of each fermentation run.

Extraction of LPS

LPS was extracted by the phenol water (PW) method as described by Westphal and Luderitz;[13] by the phenol-chloroform-petroleum (PCP) method as described by Galanos *et al.*,[14] and by the Triton-Mg^{2+} method described by Uchida and Mizushima.[15] All native LPS (5 mg/ml) were freed from protein contamination by treating with proteinase K 20 μg/ml at 65°C for 2 h. Proteinase K was removed by two washes with pyrogen-free water (PFH_2O) at 100000 *g*. Batches of de-ionised and sodium salt form LPS were prepared by the method of Galanos as described by Hancock and Poxton.[16] Further native enterobacterial LPS used in the TNF induction assays were obtained from PW or PCP extracts prepared by F. McLoughlin, Department of Medical Microbiology, Edinburgh University Medical School.

Polyacrylamide gel electrophoresis

Polyacrylamide gels (14% without SDS) were prepared with the buffer system of Laemmli.[17] Each extract was dissolved to a concentration of LPS 1 mg/ml in the SDS-2-mercaptoethanol solubilisation buffer of Laemmli[17] and heated at 100°C for 3 min. Samples (20 μl) were applied to a non-SDS polyacrylamide 14% gel. To visualise LPS, gels were oxidised with periodate and stained with silver by a method modified from that of Tsai and Frasch.[16,18] Gels were stained for protein by the Coomassie Blue method as described by Hancock and Poxton.[16]

Chemical analysis of LPS

Neutral sugars were quantified by the colorimetric method of Dubois *et al.*[19] The phosphorus content was measured by the method of Chen *et al.*[20] Bacteroides LPS were dephosphorylated by hydrofluoric acid as described by Beckmann *et al.*[9] and the KDO content of

Table I. Bacterial strains

Species	Strain no.	Origin or source
B. fragilis	MPRL 1669	Appendix, NCTC 9343
B. fragilis	MPRL 1504	Wound
B. vulgatus	MPRL 1985	Blood
B. thetaiotaomicron	MPRL 1720	Faeces
B. caccae	MPRL 1555	Wound
B. uniformis	MPRL 1721	?, ATCC 8492
B. ovatus	MPRL 1709	?, ATCC 8483
E. coli O18:K$^-$ (bort)	MPRL 1274	Dr A. S. Cross, Walter Reed Army Institute for Research, Washington DC, USA

NCTC, National Collection of Type Cultures, PHLS, Colindale Avenue, London; ATCC, American Type Culture Collection, Rockville, MD, USA; MPRL, departmental stock culture.

Table II. Details of LPS library

Strain	Growth medium	Culture volume (L)		Yield from different extraction methods*		
				PW	PCP	Triton
B. fragilis NCTC 9343	VT	16	1	6·87	6·87	6·87
			2	41·4	227·5	175·6
			3	0·60	3·31	2·56
B. fragilis NCTC 9343	PPY	15	1	2·34	2·39	2·36
			2	37·4	99·2	47·2
			3	1·60	4·42	2·00
B. fragilis MPRL 1504	PPY	15	1	2·85	2·57	2·50
			2	63·7	64·5	47·6
			3	2·24	2·51	1·91
B. vulgatus MPRL 1985	PPY	15	1	2·42	2·47	2·44
			2	90·7	96·9	53·2
			3	3·74	3·92	2·18
B. thetaiotaomicron MPRL 1720	PPY	15	1	3·23	3·67	3·77
			2	29·8	103·8	83·0
			3	0·92	2·83	2·20
B. caccae MPRL 1555	PPY	15	1	7·40	7·63	7·50
			2	20·3	98·6	20·7
			3	0·27	1·29	0·28
B. uniformis MPRL 1721	PPY	15	1	6·75	5·21	5·60
			2	25·8	213·4	57·7
			3	0·38	4·24	1·03
B. ovatus MPRL 1709	PPY	15	1	3·04	3·00	2·97
			2	54·9	124·0	73·3
			3	0·81	4·14	2·47
E. coli O18:K$^-$ MPRL 1275	NB Aerobic	15	1	7·63	—	—
			2	169·8	—	—
			3	2·23	—	—
E. coli O18:K$^-$ MPRL 1275	NB Anaerobic	16	1	1·18	—	—
			2	47·5	—	—
			3	4·04	—	—

VT, Van Tassell and Wilkins' medium; PPY, proteose-peptone yeast extract broth; NB, nutrient broth; PW, phenol-water; PCP, phenol-chloroform-petroleum.
* 1, dry weight of cells (g); 2, yield of LPS (mg); 3, % yield.

all LPS was determined by a modified thiobarbiturate assay.[16] Protein was measured by the Folin assay.[21]

Mouse lethality: galactosamine model

D-Galactosamine (12 mg/mouse) was administered intraperitoneally to groups of three 6–8-week-old male C57 black mice just before intraperitoneal administration of LPS in the range 5 ng–5 μg. Survival was recorded up to 24 h. Animal experiments were performed in accordance with Home Office guidelines.

Limulus amoebocyte lysate (LAL) assay

LPS samples were diluted to ranges of 50–0·5 ng/ml in PFH_2O. Each sample (50 μl) was added in duplicate to wells of microtitration plates (Greiner). Chromogenic LAL reagent (Coatest Endotoxin, Chromogenix, Sweden) was added to each well through a transfer plate to ensure that each well received the LAL reagent at the same time. To minimise error through temperature gradients the outer wells of the plate were left unfilled. The plate was read kinetically every 19 s at

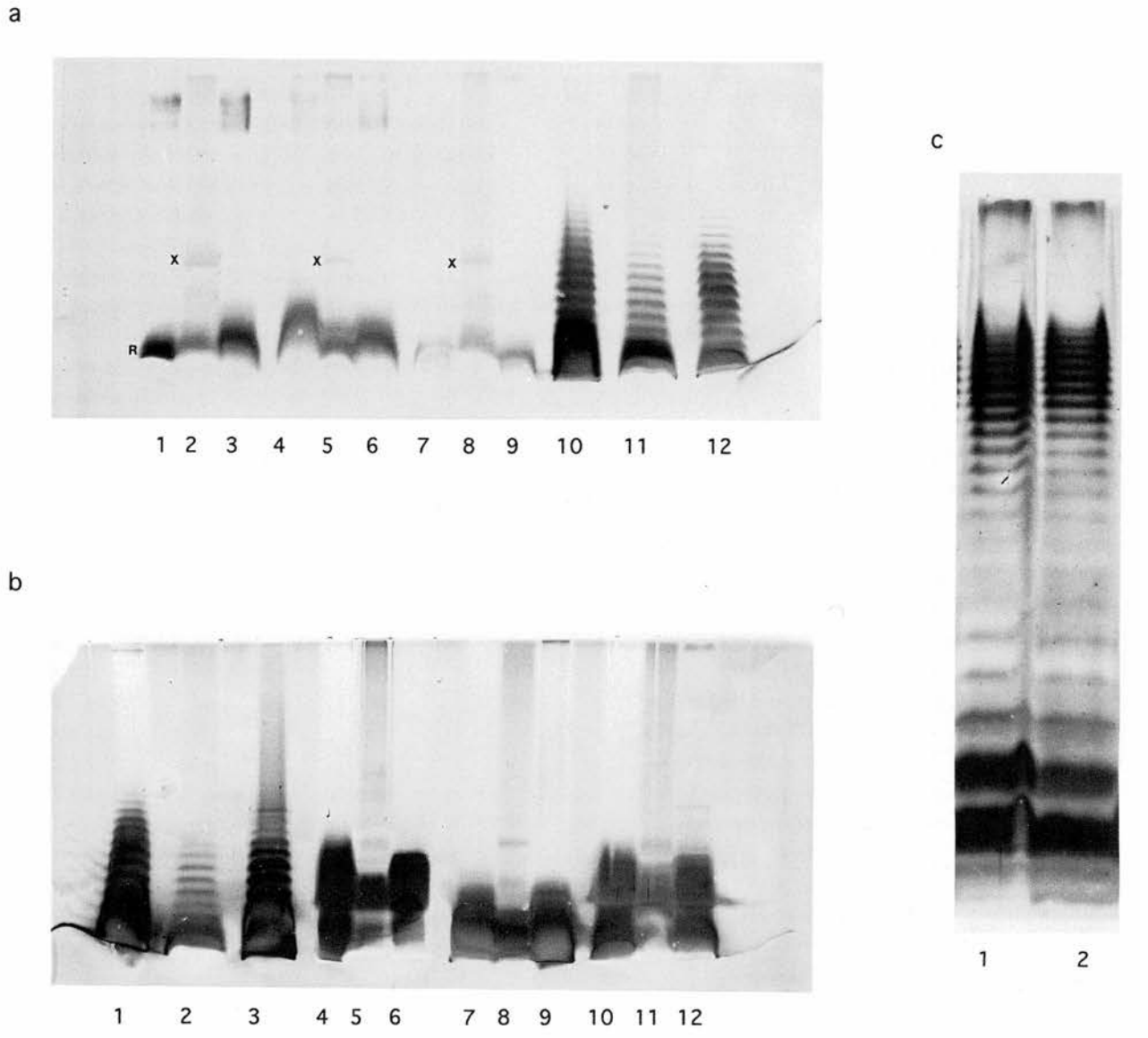

Fig. 1. Silver stain profile of LPS library. R, position of rough LPS; X, band present in PCP preparations. **a**: track **1**, NCTC 9343 VT PW; **2**, NCTC 9343 VT PCP; **3**, NCTC 9343 VT TRITON; **4**, NCTC 9343 PPY PW; **5**, NCTC 9343 PPY PCP; **6**, NCTC 9343 PPY TRITON; **7**, MPRL 1504 PPY PW; **8**, MPRL 1504 PPY PCP; **9**, MPRL 1504 PPY TRITON; **10**, MPRL 1985 PPY PW; **11**, MPRL 1985 PPY PCP; **12**, MPRL 1985 PPY TRITON. **b**: track **1**, MPRL 1720 PPY PW; **2**, MPRL 1720 PPY PCP; **3**, MPRL 1720 PPY TRITON; **4**, MPRL 1555 PPY PW; **5**, MPRL 1555 PPY PCP; **6**, MPRL 1555 PPY TRITON; **7**, MPRL 1721 PPY PW; **8**, MPRL 1721 PPY PCP; **9**, MPRL 1721 PPY TRITON; **10**, MPRL 1709 PPY PW; **11**, MPRL 1709 PPY PCP; **12**, MPRL 1709 PPY TRITON. **c**: track **1**, *E. coli* O18:K$^-$ LPS grown aerobically; **2**, *E. coli* O18:K$^-$ LPS grown anaerobically.

405 nm (reference background 650 nm) for 90 min in a Thermomax plate reader (Molecular Devices) at 37°C. The replicate mean onset time for test samples was standardised against an endotoxin of known potency (*E. coli* O111:B4, Coatest Kit endotoxin standard, Chromogenix).

LPS-induced TNF secretion

Mononuclear leucocytes (MNL: *c.* 30% monocytes) from freshly collected human buffy coats (obtained from the Blood Transfusion Service, Edinburgh) were prepared by sedimentation on lymphocyte separation medium (ICN Flow). Cells (8×10^6/ml) were cultured in RPMI 1640 (Blood Transfusion Service, Edinburgh) supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 1 mM L-glutamine and fetal calf serum (FCS) 10% in the presence or absence of varying (ng) concentrations of LPS. Culture supernates were collected after 4 h and stored at −20°C for TNF determination.

Determination of TNF content

The L929 mouse fibroblast cell line, which is sensitive to TNF, was cultured in growth medium—MEM (Sigma) containing FCS 5% supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml and 1 mM L-glutamine, and maintained by splitting 1 in 10 twice weekly. Cells were dislodged by a solution of trypsin 0·05%, EDTA 0·02% to avoid cell clumping, washed and resuspended in a growth medium to

Table III. Estimation of carbohydrate, phosphorus and KDO content in native LPS samples

LPS sample/medium	Extraction method	CHO (μg/mg dry wt)	P (μg/mg dry wt)	KDO (μg/mg dry wt)
B. fragilis NCTC 9393/VT	PW	415·8	16·4	7·8
	PCP	614·4	33·1	0·8
	Triton	229·2	29·3	3·5
B. fragilis NCTC 9343/PPY	PW	172·2	39·2	3·8
	PCP	90·8	33·6	1·4
	Triton	147·8	49·0	2·1
B. fragilis MPRL 1504/PPY	PW	198·2	55·3	6·2
	PCP	120·8	72·4	0·5
	Triton	130·0	28·5	3·7
B. vulgatus MPRL 1985/PPY	PW	267·6	78·9	2·7
	PCP	67·4	47·4	0·9
	Triton	197·2	37·8	2·6
B. thetaiotaomicron MPRL 1720/PPY	PW	363·8	54·2	—
	PCP	43·8	47·4	0·4
	Triton	262·4	79·6	4·0
B. caccae MPRL 1555/PPY	PW	240·2	54·3	—
	PCP	29·6	33·8	—
	Triton	148·6	24·9	—
B. uniformis MPRL 1721/PPY	PW	221·6	102·0	—
	PCP	53·0	38·2	—
	Triton	173·4	85·5	—
B. ovatus MPRL 1709/PPY	PW	281·0	43·1	5·7
	PCP	110·8	39·6	1·1
	Triton	178·0	41·6	2·4
E. coli O18:K⁻/ NB aerobic	PW	254·8	36·5	2·2
E. coli O18:K⁻/ NB anaerobic	PW	260·0	34·0	2·4

PW, phenol-water; PCP, phenol-chloroform-petroleum; VT, Van Tassell and Wilkins' medium; PPY, proteose-peptone yeast extract broth; NB, nutrient broth; CHO, carbohydrate; P, phosphorus; —, not done. All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for each experiment.

3×10^5 cells/ml. Cells were dispensed in flat-bottomed microtitration plates (Greiner) at 100 μl/well and incubated in CO_2 5% at 37°C for 20 h. The growth medium was then aspirated, discarded and replaced with 100 μl/well of assay medium—MEM containing FCS 5% supplemented with 1 mM glutamine and actinomycin-D (to stop further cell growth without killing the cells) 2 μg/ml. To experimental wells, 100 μl of test supernate diluted 1 in 5 in assay medium was added. A standard of recombinant TNF (National Institute for Biological Standards and Controls) diluted serially 1 in 5 from a starting concentration of 1000 IU/ml and wells without TNF were included. Plates were sealed and incubated in CO_2 5% at 37°C for 24 h. The medium was then discarded and replaced with filtered (0·22 μm) crystal violet solution (crystal violet 0·5% in methanol 20% v/v in distilled water) 100 μl/well which stains surviving cells. After 2 min, the plates were washed vigorously under tap water and dried. The crystals were dissolved by addition of 100 μl of acetic acid 20% v/v to all wells and the plate was read at 585 nm on a Vmax plate reader (Molecular Devices). The content of TNF was calculated relative to the standard curve.

Results

Comparison of different extraction methods

The yield of native LPS obtained with each extraction method is shown in table II. The PCP extraction method gave the highest yield of LPS for each organism, whereas the PW method gave the lowest yield for most organisms. Those organisms that gave a high yield of dry cells were very mucoid and the yield of LPS was generally poorer.

Polyacrylamide gels were run and stained for LPS and protein. Contaminating protein was found in bacteroides LPS extracted by all three methods, especially those extracted by the PCP and Triton methods (data not shown). LPS extracts were assayed for protein content:[21] in PCP extracts protein content ranged from undetectable to > 200 μg/mg dry weight; in all Triton preparations, with the exception of that from *B. vulgatus* MPRL 1985 in which none was detected, protein contamination was in the range 60–500 μg/mg dry weight; and none was detectable in the PW extracts.

All LPS preparations were then treated with proteinase K. On repeating the protein assays, none was detected and no Coomassie Blue-staining material was

Table IV. LPS-induced mouse lethality (galactosamine model)

LPS sample	Number of survivors after				
	5 ng	50 ng	5 μg	10 μg	20 μg
B. fragilis NCTC 9343 VT PW	—	—	3/3	1/3	0/3
B. fragilis NCTC 9343 PPY PW	—	—	3/3	2/3	1/3
B. fragilis MPRL 1504 PW	—	—	3/3	3/3	2/3
B. vulgatus MPRL 1985 PW	—	—	3/3	3/3	0/3
B. thetaiotaomicron MPRL 1720 PW	—	—	3/3	3/3	1/3
B. caccae MPRL 1555 PW	—	—	3/3	3/3	1/3
B. uniformis MPRL 1721 PW	—	—	3/3	3/3	3/3
B. ovatus MPRL 1709 PW	—	—	3/3	3/3	3/3
B. fragilis NCTC 9343 PPY PCP	—	—	3/3	3/3	3/3
B. fragilis NCTC 9343 PPY Triton	—	—	3/3	3/3	3/3
* *B. fragilis* NCTC 9343 PPY PW	—	—	3/3	2/3	0/3
* *B. fragilis* NCTC 9343 PPY PCP	—	—	3/3	3/3	3/3
* *B. fragilis* NCTC 9343 PPY Triton	—	—	3/3	3/3	3/3
E. coli O18:K⁻ aerobic PW	3/3	0/3	0/3	—	—
E. coli O18:K⁻ anaerobic PW	3/3	0/3	0/3	—	—

All LPS are native samples unless indicated.
* Sodium salt form LPS; see table III for other abbreviations.

Table V. Endotoxic activities of sodium salt and native LPS (5 ng/ml) samples

LPS sample	LAL IU/ml	
	Sodium salt form	Native form
(i) PW extracts		
B. fragilis NCTC 9343 VT	75·4	617·4
B. fragilis NCTC 9343 PPY	48·7	227·8
B. fragilis MPRL 1504	136·2	1863
B. vulgatus MPRL 1985	3·3	15·6
B. thetaiotaomicron MPRL 1720	1·0	4·1
B. caccae MPRL 1555	67·4	234·9
B. uniformis MPRL 1721	0·9	13·7
B. ovatus MPRL 1709	12·5	4·3
E. coli O18:K⁻ aerobically grown PW	6·8	62·2
E. coli O18:K⁻ anaerobically grown PW	2·2	31·7
(ii) PCP extracts		
B. fragilis NCTC 9343 VT	1·1	0·5
B. fragilis NCTC 9343 PPY	4·9	0·6
B. fragilis MPRL 1504	0·8	0·9
B. vulgatus MPRL 1985	4·2	0·4
B. thetaiotaomicron MPRL 1720	0·2	0·8
B. caccae MPRL 1555	0·2	0·1
B. uniformis MPRL 1721	7·9	20·7
B. ovatus MPRL 1709	0·1	0·6
E. coli O18:K⁻ aerobically grown PW	10·4	45·4
E. coli O18:K⁻ anaerobically grown PW	6·0	23·9
(iii) Triton extracts		
B. fragilis NCTC 9343 VT	226·2	480·4
B. fragilis NCTC 9343 PPY	455·7	71·7
B. fragilis MPRL 1504	1020	704
B. vulgatus MPRL 1985	34·6	11·1
B. thetaiotaomicron MPRL 1720	63·5	17·4
B. caccae MPRL 1555	6·0	50·6
B. uniformis MPRL 1721	22·2	42·3
B. ovatus MPRL 1709	124·4	46·3
E. coli O18:K⁻ aerobically grown PW	9·2	83·6
E. coli O18:K⁻ anaerobically grown PW	8·8	42·3

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for all assays. See table III for abbreviations.

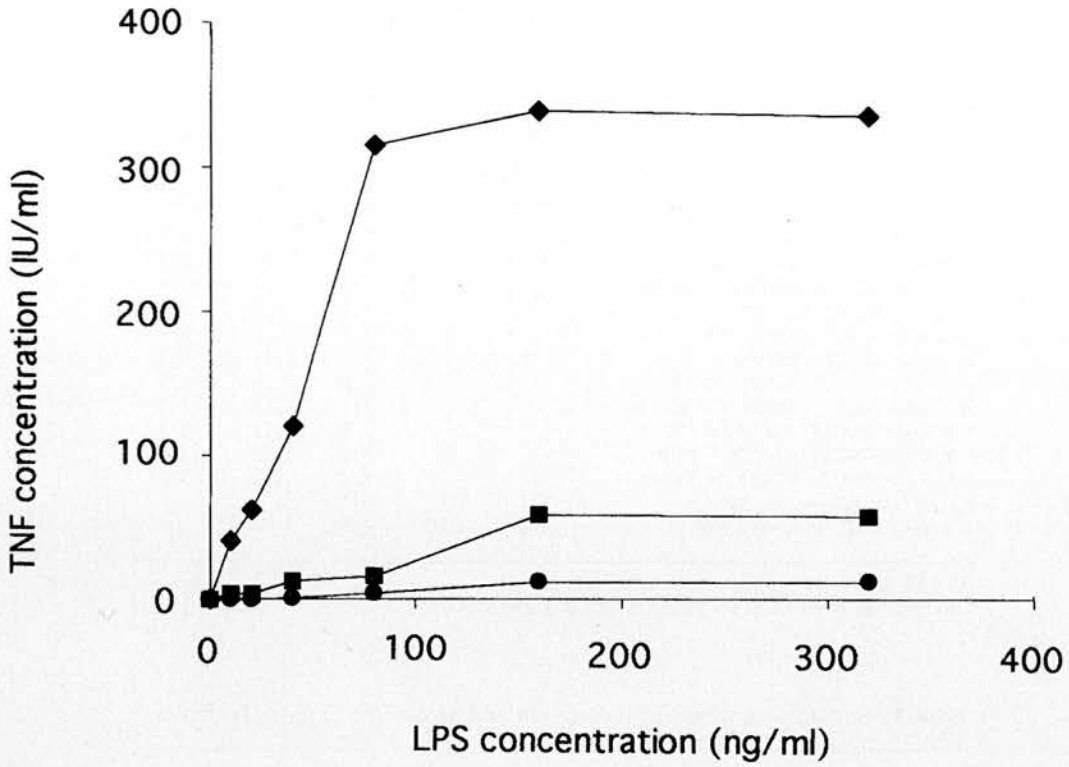

Fig. 2. TNF concentration (IU/ml) from human mononuclear leucocytes (8×10^6 cells/ml) after stimulation for 4 h with *B. fragilis* NCTC 9343 PPY (■), *B. vulgatus* MPRL 1985 (●) and aerobically grown *E. coli* O18:K$^-$ (◆) native LPS samples extracted by the PW method. Each point is the mean of two readings.

observed in PAGE. LPS profiles after silver staining were identical to those before proteinase K treatment (data not shown). The following results are from LPS preparations which had been treated with proteinase K to remove protein contamination.

Fig. 1 shows LPS profiles on PAGE for each strain and extraction method after separation and silver staining. A banding pattern typical of smooth LPS was seen for *E. coli* O18:K$^-$ (gel c, tracks 1 and 2), *B. vulgatus* MPRL 1985 (gel a, tracks 10, 11 and 12) and *B. thetaiotaomicron* MPRL 1720 (gel b, tracks 1, 2 and 3). Material of high M_r was seen in *B. fragilis* LPS extracted by both the PW and Triton methods. Rough, low M_r material was seen in extracts prepared for all species by each method, and was "dumbbell shaped" for *B. caccae* and *B. ovatus* (gel b, tracks 4, 5 and 6, and 10, 11 and 12). The PCP extraction method produced a pronounced band (X) present behind the main front band (R) in all preparations. For *B. fragilis*, the PCP extraction method did not select for high M_r material. Silver stain profiles for sodium salt form LPS were identical to profiles of native forms (data not shown). LPS profiles for *E. coli* O18:K$^-$ grown aerobically and anaerobically were identical.

Chemical analysis of LPS samples

Samples of dry extract were resuspended in PFH_2O to a concentration of 5 mg/ml and assayed for carbohydrate, phosphorus and KDO content. The relative amounts of carbohydrate, phosphorus and KDO are shown in table III. *Bacteroides* spp. showed a negligible amount or no KDO content before treatment with hydrofluoric acid. The PW extraction method selected for the greatest amount of carbohydrate and KDO material and the PCP extraction method for the least. Phosphorus levels were more uniform among all extraction methods. No difference was observed between LPS extracts from *E. coli* O18:K$^-$ grown in different atmospheres.

LPS induced mouse lethality (galactosamine model)

E. coli O18:K$^-$ LPS was lethal to all mice at 50 ng/mouse (table IV) whereas *B. fragilis* NCTC 9343 VT PW-LPS and *B. vulgatus* MPRL 1985 PW-LPS were only lethal to mice at 20 μg/mouse. No difference in lethality was seen for native and sodium salt form *B. fragilis* NCTC 9343 VT PW-LPS. Mice were not killed by the maximum dose of *B. fragilis* LPS extracted by PCP or Triton, nor by *B. uniformis* and *B. ovatus* LPS extracted by PW.

Reactivity of LPS samples in LAL assay

Endotoxic activities in the LAL assay of native and sodium salt form LPS are illustrated in table V (i), (ii) and (iii) for each extraction method. On a weight for weight basis, the greatest activity was seen in the PW extracts and the least activity seen in the PCP extracts. Most native PW-LPS were more active than sodium

Table VI. Measured TNF concentration from human MNL (8×10^6 cells/ml) after stimulation for 4 h with native and sodium salt form LPS (50 ng/ml) from *E. coli* O18:K⁻ and *Bacteroides* spp. extracted by three different methods

LPS sample	Extraction method	TNF concentration (IU/ml)	
		Native LPS	Sodium salt LPS
B. fragilis NCTC 9343 VT	PW	23·16	11·92
	PCP	0·30	0·71
	Triton	2·99	1·07
B. fragilis NCTC 9343 PPY	PW	18·81	4·60
	PCP	2·53	1·78
	Triton	0·51	0·31
B. fragilis MPRL 1504	PW	4·93	2·51
	PCP	0·42	0·25
	Triton	0·99	0·47
B. vulgatus MPRL 1985	PW	2·58	0·72
	PCP	0·15	0·51
	Triton	0·39	0·31
B. thetaiotaomicron MPRL 1720	PW	6·40	6·47
	PCP	0·03	0·43
	Triton	0·17	0·49
B. caccae MPRL 1555	PW	13·89	15·25
	PCP	0·22	0·74
	Triton	2·48	2·44
B. uniformis MPRL 1721	PW	10·10	35·34
	PCP	4·81	4·81
	Triton	10·87	5·09
B. ovatus MPRL 1709	PW	8·66	13·89
	PCP	1·44	5·37
	Triton	1·25	2·32
E. coli O18:K⁻ aerobically grown	PW	94·61	43·21
E. coli O18:K⁻ anaerobically grown	PW	99·00	43·21
E. coli O6	PW	59·00	—
E. coli O12	PW	58·20	—
E. coli O15	PW	68·96	—
K. pneumoniae M10B	PW	48·21	—
E. coli J5	PCP	36·63	—
E. coli K12	PCP	49·90	—
S. minnesota Ra	PCP	31·68	—
S. minnesota Rb	PCP	96·70	—
S. minnesota Rc	PCP	49·90	—
S. minnesota Rd	PCP	78·45	—
S. minnesota Re	PCP	50·48	—

All assays were carried out on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for each assay. See table III for abbreviations.

salt PW-LPS. Approximately 70% of native PCP-LPS are more active than sodium salt PCP-LPS. No significant difference was seen between the native and sodium salt form LPS extracted by Triton. LPS from *E. coli* O18:K⁻ grown aerobically was more active than LPS from *E. coli* O18:K⁻ grown anaerobically by a factor of two. *B. fragilis* MPRL 1504 PW-LPS was more active than *E. coli* O18:K⁻ LPS by a factor of 30, *B. fragilis* NCTC 9343 VT PW-LPS by a factor of 10, *B. fragilis* NCTC 9343 PPY PW-LPS by a factor of four and *B. caccae* MPRL 1555 PW-LPS by a factor of four. LPS from all other *Bacteroides* spp. were less active than that from *E. coli* O18:K⁻. A difference in activity for *B. fragilis* NCTC 9343 LPS grown in different media was also seen.

LPS-induced TNF secretion from human buffy coats

The amount of TNF induced by different LPS from human MNL (8×10^6 cells/ml) at several different concentrations is shown in fig. 2. TNF appears to reach a maximal level at *c.* 160 ng of LPS for all those tested. Based on the data from fig. 2, an LPS concentration of 50 ng/ml was selected to compare a larger panel of LPS from other *Bacteroides* spp. and enterobacteria (table VI). This value was chosen as it was in the middle of the linear part of both the dose response curve and the standard curve of the assay. This would not show the maximum induction capacity of the LPS but was a compromise for comparing a large number of LPS samples. *E. coli* O18:K⁻ LPS stimulated the most TNF production with no difference in TNF production between LPS from *E. coli* O18:K⁻ grown aerobically or anaerobically. On a weight for weight basis, PW-LPS stimulated the greatest TNF production. Comparing PW-LPS, bacteroides LPS induced TNF much less than did *E. coli* O18:K⁻ LPS, with the most active (*B. fragilis* NCTC 9343 VT) being four times less active than *E. coli* O18:K⁻ and the least active (*B. vulgatus* MPRL 1985)

Table VII. TNF concentration from human MNL and endotoxic activity after stimulation with LPS from selected *Bacteroides* spp. and *E. coli* O18:K⁻ LPS relation to KDO content

LPS sample	Extraction method	TNF stimulation (IU/ng of KDO)	LAL activity (EU/ng of KDO)
B. fragilis NCTC 9343 VT	PW	59·38	1583·08
	PCP	7·16	10·69
	Triton	17·3	2784·93
B. fragilis NCTC 9343 PPY	PW	46·11	1191·11
	PCP	35·11	8·44
	Triton	4·90	693·24
B. fragilis MPRL 1504	PW	15·85	5985·54
	PCP	17·42	37·38
	Triton	5·38	3846·99
B. vulgatus MPRL 1985	PW	19·11	115·48
	PCP	3·41	7·93
	Triton	3·00	84·67
B. thetaiotaomicron MPRL 1720	PW	—	—
	PCP	1·55	42·61
	Triton	8·64	867·91
B. ovatus MPRL 1709	PW	30·37	14·98
	PCP	25·89	4·68
	Triton	10·43	385·5
E. coli O18:K⁻ aerobic	PW	502·16	572·32
E. coli O18:K⁻ anaerobic	PW	485·60	261·15

See table III for abbreviations.

being 37 times less active than *E. coli* O18:K⁻. TNF production with other enterobacterial LPS tested was of the same level or less than that with *E. coli* O18:K⁻ LPS, all being more active than bacteroides LPS. Sodium salt form *E. coli* O18:K⁻ LPS had half the activity of native form *E. coli* O18:K⁻ LPS. Generally for bacteroides LPS there was no clear distinction in activity between sodium salt and native form LPS.

Reactivity of LPS samples on the basis of KDO concentration

All the above assays were done on a weight for weight basis. However, because endotoxicity is likely to depend on lipid A concentration, it was decided to re-evaluate some of the results of TNF induction and LAL activity relative to KDO concentration in an attempt to compare lipid A levels—on the assumption (which could well be false) that lipid A is proportional to KDO in all species.

The TNF concentration from human buffy coats expressed as IU/ng of KDO and endotoxic activity in LAL expressed as EU/ng of KDO after stimulation with selected bacteroides and *E. coli* O18:K⁻ LPS is presented in table VII. For the TNF concentrations measured, this shows that the low activities seen previously on a weight for weight basis (table VI) were due in part to less KDO being present. However, LAL activity for PCP-extracted LPS was still low when the results were represented/ng of KDO.

Discussion

This study has shown that an aqueous phenol (PW) extract of bacteroides LPS is significantly different both in terms of chemical composition and biological activity from the PCP or Triton products. The three methods were selected in an attempt to produce products of different composition. The classical PW method was likely to select for higher M_r, less hydrophobic material, the PCP method for rough-form, more hydrophobic material, and the Triton-Mg^{2+} method was chosen as a milder, less selective method. It is well accepted that enterobacterial LPS is heterogeneous in M_r even when pure. We acknowledge that the bacteroides "LPS" used in this study may be heterogeneous both in M_r and in molecular composition. The degree of different molecular species of LPS and other co-purifying carbohydrate, due to the probable subpopulation structure of our cultures,[3] is by no means certain. The biological activity of enterobacterial LPS is thought to depend on its solubility[22] and, for this reason, the native and the standardised, soluble sodium salt forms were prepared from electrodialysed material.

Our results agree with previous studies[7] that in a mouse lethality model bacteroides LPS is *c.* 5000-fold less active than LPS from *E. coli*. In past studies,[7,23,24] bacteroides LPS has been quoted as being 100–1000-fold less biologically active *in vitro*, depending on the assay, than LPS from enterobacteria. Our results for bacteroides LPS extracted by the PCP or Triton method agree with this. However, our results for LPS

extracted by the PW method disagree with the accepted view.

Tumour necrosis factor-alpha (TNF) is considered to be the pivotal cytokine in the host response to endotoxin. In the TNF induction assay, *E. coli* O18:K⁻ LPS was only four-fold more active than *B. fragilis* LPS, seven-fold more than *B. caccae* LPS and up to 50-fold more than the other bacteroides LPS. In the LAL assay, which has questionable value for the in-vivo situation, the same LPS that were the most active in the TNF-induction assay (from *B. fragilis* and *B. caccae*) were 30–40-fold more active than *E. coli* O18:K⁻ LPS, with *E. coli* O18:K⁻ LPS being *c.* 10-fold more active than the rest of the bacteroides LPS tested. The two-fold difference in activity in the LAL assay for LPS from *E. coli* O18:K⁻ grown aerobically and anaerobically is unclear. Our results also show that solubility (native *versus* sodium salt form LPS) does not play an important role in biological activity as has been described previously for enterobacterial LPS;[22] in most cases the sodium salt form was of lower activity. Growth in the defined van Tassell and Wilkins' medium was included because we have found recently that growth in this medium converts several *B. fragilis* strains, including NCTC 9343, from being sensitive to the lytic action of normal human serum to being resistant. This may be due to a change in expression of certain surface polymers such as LPS.[25] The results show some differences between the two growth media in respect to both chemical composition and biological activities.

The possibility that some contaminant may be present in the LPS preparations from *Bacteroides* spp. must be considered. In this connection we found no contaminating organisms when culturing the organisms for extraction and the identity of each strain of *Bacteroides* was confirmed before the start of the study. Furthermore, PW-LPS samples from several *Bacteroides* spp. prepared by different individuals at different times in our laboratory were tested alongside our own samples in the LAL and TNF induction assay. They were found to produce nearly identical results (data not shown). Higher biological activity in the PW preparations cannot be due to a biologically active protein as protein levels were negligible after purification (table III). Biological activity also cannot be due to any contaminating proteinase K as all bacteroides LPS had been treated similarly with proteinase K.

Some PW preparations of bacteroides LPS are clearly more active than others, e.g., *B. fragilis* NCTC 9343 VT/PPY and *B. caccae* MPRL 1555. Chemical analysis does not explain why the LPS from some species are more active than from others. It does show that more carbohydrate and KDO is present in the PW preparations. The amount of KDO destroyed or not released by hydrofluoric acid treatment is not known. Those species that had a low reactivity in the LAL and TNF bioassays (*B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720) had a smooth LPS profile on PAGE (fig. 1). This is in apparent contradiction to the view that aqueous phenol preferentially extracts smooth-form LPS. Moreover it is surprising that those strains which are obviously smooth (e.g., *B. vulgatus*) are less endotoxic.

In the light of the great variation found in the chemical analyses, PAGE profiles and biological activities of the various preparations, and the knowledge that several different LPS-like molecules have been identified by monoclonal antibodies,[26] the suggestion that different extraction methods selectively produce different populations of LPS or perhaps lipid-linked capsular polysaccharides is strong. In nature all of these different molecular species, if expressed, might contribute towards a range of "endotoxic" phenomena. Therefore, it is essential to be able to purify the various components of this heterogeneous "LPS" population and relate them to biological function.

The role of bacteroides LPS in endotoxic shock remains to be defined. We have shown that PW-extracted LPS has a higher biological activity than previously reported.[7,24] How these chemically extracted "LPS" relate to what is found *in vivo* also needs to be defined. When we consider that *Bacteroides* spp. are in vast excess of *E. coli* in the gut and may have higher biological activity than previously thought, the bacteroides LPS may play an important but neglected role in endotoxic shock.

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Tumor Necrosis Factor Induction by an Aqueous Phenol-Extracted Lipopolysaccharide Complex from *Bacteroides* Species

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The stimulation of macrophages and monocytes by lipopolysaccharide (LPS) results in the secretion of tumor necrosis factor (TNF), a cytokine which is thought to play a pivotal role in subsequent host responses. Its induction is thought to be facilitated by the binding of complexes of LPS and LPS-binding protein to CD14. The LPS of *Bacteroides* species was considered a weak endotoxin; however, in a recent study we have shown that the biological activity and chemical composition of the LPS from *Bacteroides* species are dependent on the extraction method. The present study determines the capacity of LPS extracted by aqueous phenol (the method for producing an LPS of high endotoxic activity) from four species of *Bacteroides* to induce TNF. Induction was investigated from human mononuclear leukocytes (MNL), THP-1 cells (with and without enhancement by vitamin D_3 for CD14), and peritoneal macrophages from C3H/HeJ (LPS nonresponder) and C3H/HeN (LPS responder) mice. *Escherichia coli* O18K$^-$ LPS, a typical smooth LPS of heterogeneous molecular mass, was used as a control throughout. The stimulation of TNF production by *E. coli* LPS was between two- and fourfold more than that by *Bacteroides* LPS in MNL, in THP-1 cells (with enhancement for CD14), and in peritoneal macrophages from C3H/HeN mice. In THP-1 cells (without enhancement for CD14), there was no significant difference in TNF production between *E. coli* and *Bacteroides* LPSs. In peritoneal macrophages from C3H/HeJ mice, *E. coli* LPS stimulated no TNF production, but there was no significant difference in TNF production from peritoneal macrophages from C3H/HeJ and C3H/HeN mice by *Bacteroides* LPS. In all cell populations, there was a peak of TNF production after approximately 4 h of stimulation with all LPSs tested. However, other peaks of TNF production were seen in MNL and THP-1 cells (with enhancement for CD14) after stimulation with *E. coli* LPS only. In stimulation assays in which *Bacteroides* LPS was together with but in excess of *E. coli* LPS, it was found that TNF production from MNL and THP-1 cells (with and without enhancement for CD14) was comparable to that of *Bacteroides* LPS alone and not *E. coli* LPS alone. An anti-CD14 monoclonal antibody did not inhibit *Bacteroides* LPS-stimulated TNF production. However, *E. coli* LPS-stimulated TNF release was inhibited by an anti-CD14 monoclonal antibody, most noticeably in MNL and THP-1 cells (with enhancement for CD14). We conclude that *Bacteroides* LPS can mask the effects of *E. coli* LPS when present in excess, can produce only one peak of TNF production, and activates mononuclear cells by a pathway not dependent on CD14.

The gram-negative bacterium *Bacteroides fragilis* is the most frequently isolated anaerobic species in many infectious processes, including intra-abdominal abscesses. Such infections usually arise from fecal contamination. Anaerobes constitute approximately 99% of the fecal bacterial mass; of this amount, *Bacteroides* species (formerly members of the *B. fragilis* group) account for 20 to 30% of species isolated (6). The genus *Bacteroides* constitutes the opportunistic pathogen *B. fragilis* and several other pathogenic and nonpathogenic species: *B. vulgatus*, *B. caccae*, *B. uniformis*, *B. distasonis*, *B. thetaiotaomicron*, *B. stercoris*, *B. ovatus*, and *B. merdae*. *Bacteroides* species are numerically more predominant (by at least 10^3-fold) in the gut than members of the family *Enterobacteriaceae* such as *Escherichia coli*, thus representing a potentially vast pool of biologically active lipopolysaccharide (LPS).

Studies on LPSs from *Bacteroides* species and their biological activities have mainly concentrated on *B. fragilis* (14, 32). It is well documented that *B. fragilis* contains different fatty acids from enterobacterial LPS, is monophosphorylated, and is generally accepted as being less endotoxic (100- to 1,000-fold less, depending on the assay) (14).

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In a recent study in our laboratory, we found that the chemical composition, appearance on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and biological activity of *Bacteroides* LPS were dependent on the extraction method used (5). Comparison of three extraction methods for LPS, the aqueous phenol method (33), the phenol-chloroform-petroleum method (8), and the Triton-Mg^{2+} method (31), showed that the phenol-chloroform-petroleum and Triton-Mg^{2+} methods produced LPS of low activity, in keeping with previous studies, but the aqueous phenol method produced LPS of much higher activity. All LPS preparations were complex in PAGE, and the aqueous phenol-extracted material tended to have a greater proportion of carbohydrate and 3-deoxy-D-manno-octulosonic acid (2-keto-3-deoxyoctonic acid KDO) than the other methods and generally to contain higher-molecular-mass bands, especially those from the more biologically active species such as *B. fragilis* and *B. caccae* (5).

LPS has been implicated as an important primary mediator in the development of gram-negative sepsis, a clinical syndrome frequently associated with serious sequelae such as multiorgan failure and death (2). Several lines of evidence indicate that the biological effect of LPS may be mediated in part through the release of host cytokines (19).

Tumor necrosis factor alpha (TNF-α) is a cytokine produced primarily by monocytes and macrophages during the early phase of the host response to endotoxin (19). Recent studies

TABLE 1. Bacterial strains used[a]

Species	Strain	Source/origin
B. fragilis	MPRL 1669	Appendix/NCTC 9343
B. vulgatus	MPRL 1985	Blood
B. caccae	MPRL 1555	Wound
B. uniformis	MPRL 1721	?/ATCC 8492
E. coli O18K$^-$ (bort)	MPRL 1274	A. S. Cross, Walter Reed Army Institute for Research, Washington, D.C.

[a] MPRL, departmental stock culture; NCTC, National Type Culture Collection, United Kingdom; ATCC, American Type Culture Collection.

suggest that CD14, a myeloid cell differentiation antigen expressed primarily by monocytes and macrophages, is involved in the activation of monocytes to release large quantities of TNF-α by interacting with complexes of LPS and LPS-binding protein (12, 16, 35). Many of the sequelae of gram-negative infection can be reproduced by the administration of TNF into animals (28).

In this study, we examine the TNF-inducing capacity of an aqueous phenol-extracted LPS complex, previously shown to be the most biologically active extract (5), from several species of *Bacteroides*, representing a range of biological activities and pathogenic potential, to induce TNF secretion. This capacity was compared with that of an *E. coli* O18K$^-$ LPS control in three cell populations. The role of CD14 in activation of cells to produce TNF was also investigated.

MATERIALS AND METHODS

Bacterial strains and culture methods. The strains used are described in Table 1. Bacteria were grown in 15- to 16-liter batches in a 20-liter-capacity fermenter (LH Engineering, Stoke Poges, United Kingdom). *Bacteroides* species were grown in proteose-peptone yeast medium (13) anaerobically at 37°C in 100% N_2. *E. coli* O18K$^-$ was grown in nutrient broth aerobically at 37°C at a pO_2 of 50%. Strict purity checks were carried out for each organism at the end of each fermentation run.

Extraction of LPSs. LPSs were extracted by the aqueous phenol method of Westphal and Luderitz (33) as described by Hancock and Poxton (10). All native LPSs (5 mg/ml) were made free from protein contamination by treatment with proteinase K (20 μg/ml) at 65°C for 2 h. Proteinase K was removed by two washes with pyrogen-free water at 100,000 × *g*. Samples of each were analyzed by PAGE on 14% polyacrylamide gels and stained with silver (10).

LPS-induced TNF secretion. (i) From human peripheral mononuclear leukocytes. Mononuclear leukocytes (approximately 30% monocytes) from freshly collected human buffy coats (obtained from the Blood Transfusion Service, Edinburgh, Scotland) were prepared by sedimentation on lymphocyte separation medium (ICN Flow). Cells (8×10^6/ml) were cultured in RPMI 1640 (Blood Transfusion Service) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM L-glutamine, and 10% fetal calf serum (FCS) in the absence or presence of various nanogram concentrations of LPS. Culture supernatants were collected after 4 h or at 1- or 2-h intervals for time course experiments and stored at −20°C for TNF content determination.

(ii) From THP-1 cells with and without enhancement for CD14. THP-1 cells (a human monocyte/macrophage cell line) were a gift from David Morrison, University of Kansas Medical Center, Kansas City. Cells were grown routinely in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM L-glutamine, and 10% FCS at 37°C in 5% CO_2. To induce expression of CD14, cultures were grown in the presence of 0.1 μM 1,25-dihydroxyvitamin D_3 (Sigma) for 72 h. Cells (2×10^6/ml) were cultured in RPMI 1640 supplemented as before in the absence or presence of various nanogram concentrations of LPS. Culture supernatants were collected after 4 h or at 2-h intervals for time course experiments and stored at −20°C for TNF content determination.

(iii) From peritoneal macrophages from LPS responder and nonresponder mice. The LPS nonresponder mice, strain C3H/HeJ, were obtained from Harlan Olac, Germany. The LPS responder mice, strain C3H/HeN, were obtained from Harlan Olac, England. Sodium thioglycolate (1 ml of 10% [wt/vol] in distilled H_2O) was injected intraperitoneally to each mouse 4 days prior to collection of peritoneal macrophages. To collect the macrophages, the mice were killed by cervical dislocation, and the peritoneal cavity was washed several times with 5-ml quantities of RPMI 1640. After being washed once in RPMI 1640, cells were resuspended to 2×10^6/ml in RPMI 1640 supplemented as before, and the assay carried out as described above.

Inhibition of TNF secretion with an anti-CD14 MAb. The CD14 mouse immunoglobulin G clone BA8 monoclonal antibody (MAb) was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland. The CD14 MAb was serially diluted 1:2 in RPMI 1640 and added to cell preparations (as described above) so that the starting concentration was 75 μg/ml. Cell preparations and MAb were preincubated for 30 min at 37°C on 5% CO_2 before addition of an appropriate dilution of LPS and reincubation as before. Culture supernatants were harvested at 4 h and stored at −20°C for determination of TNF content.

Determination of TNF content. The L929 mouse fibroblast cell line, which is sensitive to TNF, was cultured in growth medium (minimal essential medium [Sigma] containing 5% FCS supplemented with penicillin [100 U/ml], streptomycin [100 μg/ml] and 1 mM L-glutamine) and maintained by splitting 1:10 twice weekly. Cells were dislodged by 0.005% trypsin–0.02% EDTA to avoid cell clumping, washed, and resuspended in growth medium to 3×10^5 cells per ml. Cells were dispensed in flat-bottom microplates (Greiner) at 100 μl per well and incubated at 37°C in 5% CO_2 for 20 h. The growth medium was then aspirated, discarded, and replaced with 100 μl of assay medium (minimal essential medium containing 5% FCS supplemented with 1 mM glutamine and 2 μg of actinomycin D per ml [to stop further cell growth without killing the cells]) per well. To experimental wells, 100 μl of test supernatant which had been diluted 1:5 in assay medium was added. A standard of recombinant TNF (National Institute for Biological Standards and Control, Potters Bar, United Kingdom) diluted serially 1:5 at a starting concentration of 1,000 IU/ml and wells without TNF were included. Plates were covered and incubated at 37°C in 5% CO_2 for 24 h. The medium was then discarded and replaced with, per well, 100 μl of filtered (0.22-μm-pore-size filter) crystal violet solution (0.5% crystal violet in 20% [vol/vol] methanol in distilled water), which stains surviving cells. After 2 min, the plates were washed vigorously under tap water and dried. The crystals were dissolved by addition of 100 μl of 20% (vol/vol) acetic acid to all wells, and the plate was read at 585 nm on a Vmax plate reader (Molecular Devices). The content of TNF was calculated relative to the standard curve.

RESULTS

Inter- and intra-assay variation. Because of variations between individual blood donors and between cell cultures produced on different occasions, a degree of interassay variation in the TNF assays was unavoidable. With the exception of the results in Fig. 3, which have been analyzed statistically, the results presented are means of two experiments performed with the same cell preparations on the same day. Intra-assay variation was not great, typically much less than 10% (see Fig. 3). All assays were repeated on at least two occasions with cell supernatants derived from different stimulation experiments. All results presented depict trends which have been confirmed on at least two occasions.

Effect of amount of LPS on TNF production. The effects of various nanogram amounts of LPS on TNF production were examined for *E. coli* O18K$^-$ and *B. fragilis* NCTC 9343 (Fig. 1). Because of the heterogeneity in molecular mass of the *E. coli* LPS (ladder pattern) and the complexity of the appearance on SDS-PAGE (Fig. 2) and unknown chemical structure and molecular composition of the *Bacteroides* LPS complex (5), these results are compared on a weight-for-weight basis rather than a molar basis. However, chemical analysis of the preparations, which is detailed in reference 5 and summarized here, showed that the two preparations were essentially similar in terms of phosphorus (39.2 and 34 μg/mg), KDO (3.8 and 2.4 μg/mg), and carbohydrate (172 and 260 μg/mg) for *B. fragilis* and *E. coli*, respectively. In all cell populations tested, more TNF was produced as LPS levels increased. For human mononuclear leukocytes (Fig. 1a), *E. coli* O18K$^-$ LPS produced more TNF than *B. fragilis* NCTC 9343 LPS for all amounts tested. No difference in TNF production by *B. fragilis* NCTC 9343 LPS was seen for THP-1 cells with and without enhancement for CD14 (Fig. 1b). The level of TNF production by *E. coli* O18K$^-$ LPS in THP-1 cells without enhancement for CD14 was comparable to that produced by *B. fragilis* NCTC 9343 LPS. However, the level of TNF production by *E. coli* O18K$^-$ LPS was greatly increased in THP-1 cells with enhancement for CD14 compared with *B. fragilis* NCTC 9343. No major difference in

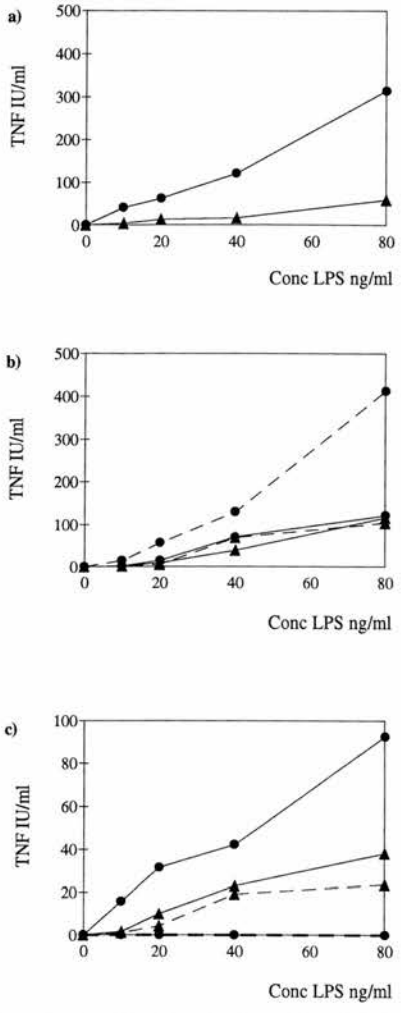

FIG. 1. TNF production after LPS stimulation for 4 h by human mononuclear leukocytes (a), THP-1 cells with (– – –) and without (—) enhancement for CD14 (b), and peritoneal macrophages from C3H/HeJ (– – –) and C3H/HeN (—) mice (c) by LPS from *E. coli* O18K$^-$ (●) and *B. fragilis* NCTC 9343 (▲). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on *y* axes.

TNF production by *B. fragilis* NCTC 9343 LPS was seen between peritoneal macrophages from C3H/HeJ (LPS nonresponder) and C3H/HeN (LPS responder) mice (Fig. 1c), but consistently there was slightly more TNF produced by the C3H/HeN cells. Note the different scales of the *y* axes. There was no TNF production observed in C3H/HeJ peritoneal macrophages stimulated by *E. coli* O18K$^-$ LPS. However, in peritoneal macrophages from C3H/HeN mice, the level of TNF production was greatly increased after stimulation by *E. coli* O18K$^-$ LPS. No difference in results was found when the assays were carried out in the presence of FCS or human serum (data not shown). The amount of TNF produced by peritoneal macrophages after stimulation by all LPSs tested was considerably lower than that produced by the other cell populations.

Time course of TNF production. Levels of TNF production by human mononuclear cells were measured at hourly intervals after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ and *B. fragilis* (Fig. 3a). The response produced by the *E. coli* LPS showed a complex periodic rise and fall, with major peaks at

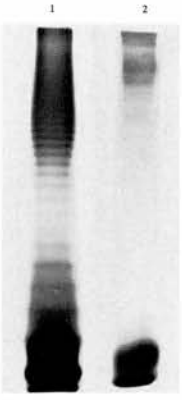

FIG. 2. Silver-stained profiles of aqueous phenol-extracted LPS preparations from *E. coli* O18K$^-$ (track 1) and *B. fragilis* NCTC 9343 (track 2) analyzed by PAGE on a 14% polyacrylamide gel. Note that the material in the upper part of track 2 does not stain strongly with silver.

between 3 and 5 h and 8 h. The *B. fragilis* LPS showed one major peak between 3 and 6 h, with a minor rise at 8 h. This type of response for *E. coli* LPS has been seen by other workers in other laboratories (9, 9a), and a second peak at 8 h is a consistent finding that has been reproduced on numerous occasions in our laboratories by several different workers. To confirm the periodicity of the TNF response to *E. coli* LPS, the experiment was repeated on four occasions with mononuclear leukocytes from different donors, sampling at 2-h intervals until 8 h and then at 20 h (Fig. 3b and c). All mean TNF values in international units per milliliter from duplicate estimations were normalized to the value for *E. coli* LPS at 4 h, which was given the arbitrary value of 100%. Statistical analysis by Student's *t* test for paired means showed that the differences between peaks and troughs for the response to *E. coli* LPS were highly significant: between 2 and 4 h, $P = 0.0027$; between 4 and 6 h, $P = 0.0032$; and between 6 and 8 h, $P = 0.0312$ ($P \leq 0.05$ is significant).

TNF induction by LPSs from other *Bacteroides* spp. and *E. coli* were also followed by sampling at 2-h intervals in other cell populations (Fig. 4). In human mononuclear leukocytes (Fig. 4a and b), LPS from *E. coli* O18K$^-$ was, depending on the occasion assayed, between two and four times as active as the most active *Bacteroides* LPS, with LPS from *B. fragilis* NCTC 9343 being the most active and that from *B. vulgatus* MPRL 1985 being the least active. For THP-1 cells without enhancement for CD14, only one peak of TNF was stimulated at 4 h for all LPSs tested. LPSs from *E. coli* O18K$^-$ and *B. uniformis* MPRL 1721 were the most active, and LPS from *B. vulgatus* was the least active (Fig. 4a). In THP-1 cells with enhancement for CD14, a peak of TNF production was seen at 4 h for all LPSs tested. However, a second peak of TNF production, larger than the first, was seen at 8 h for *E. coli* O18K$^-$ only. LPS from *E. coli* O18K$^-$ was approximately three- to fivefold more active than the most active *Bacteroides* LPS (Fig. 4b). In peritoneal macrophages from both C3H/HeJ (LPS nonresponder) and C3H/HeN (LPS responder) mice, a peak of TNF production was seen at 4 h for the LPSs tested (Fig. 4c and d), with the exception of *E. coli* O18K$^-$ in C3H/HeJ peritoneal macrophages, in which case no TNF was produced. In peritoneal macrophages from C3H/HeN mice, *E. coli* O18K$^-$ LPS was only twofold more active than *B. fragilis* NCTC 9343 LPS. The difference in activity between *B. fragilis* NCTC 9343 and *B. vulgatus* MPRL 1985 LPS was not as marked as previously seen. In most cell populations, the amount of TNF production

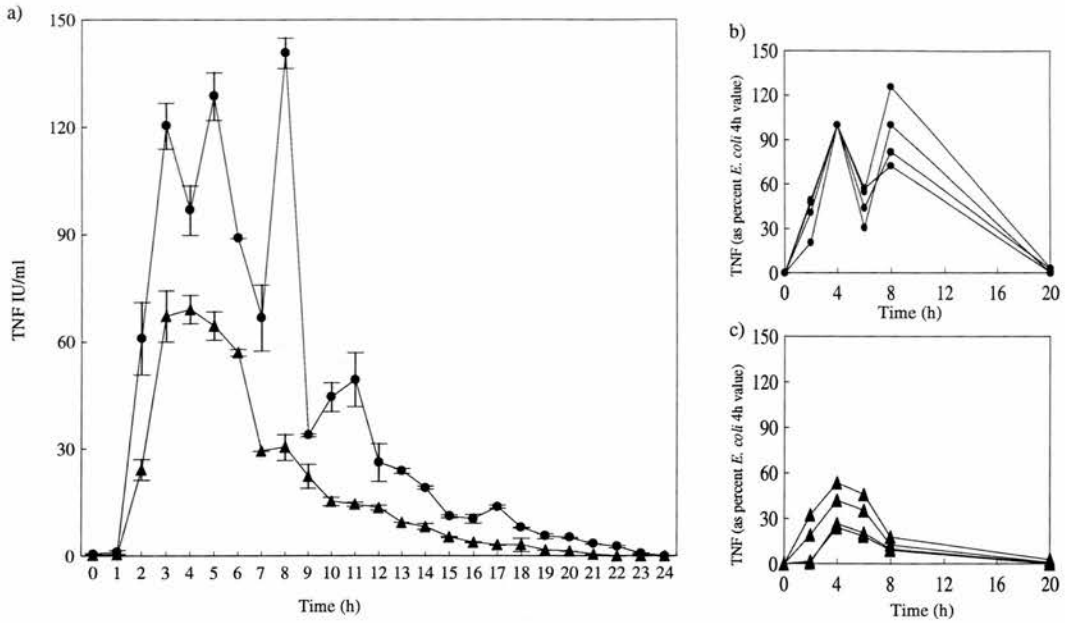

FIG. 3. Time course of TNF production after stimulation of human mononuclear leukocytes with LPS (50 ng/ml) from *E. coli* O18K$^-$ (●) or *B. fragilis* NCTC 9343 (▲). (a) Samples taken hourly over 24 h from a single experiment. TNF values are means of duplicate TNF estimations (vertical bars indicate differences between duplicate estimations). (b and c) Results from four separate experiments performed on different occasions with different cell preparations. Each point is the mean of two TNF estimations, and all values have been normalized relative to the 4-h value for *E. coli* LPS, which has been defined as 100%.

fell to zero more quickly for *Bacteroides* LPS than for *E. coli* O18K$^-$ LPS.

Effect of TNF production by *E. coli* O18K$^-$ LPS with *B. fragilis* NCTC 9343 LPS present in excess. When LPS from *B. fragilis* NCTC 9343 was in excess of a constant amount of *E. coli* O18K$^-$ LPS (40 ng/ml) in human mononuclear leukocytes and THP-1 cells with and without enhancement for CD14, TNF production was comparable to that of *B. fragilis* NCTC 9343 LPS alone and not that of *E. coli* O18K$^-$ alone (Fig. 5a to c). There appeared to be a lowering and masking effect of

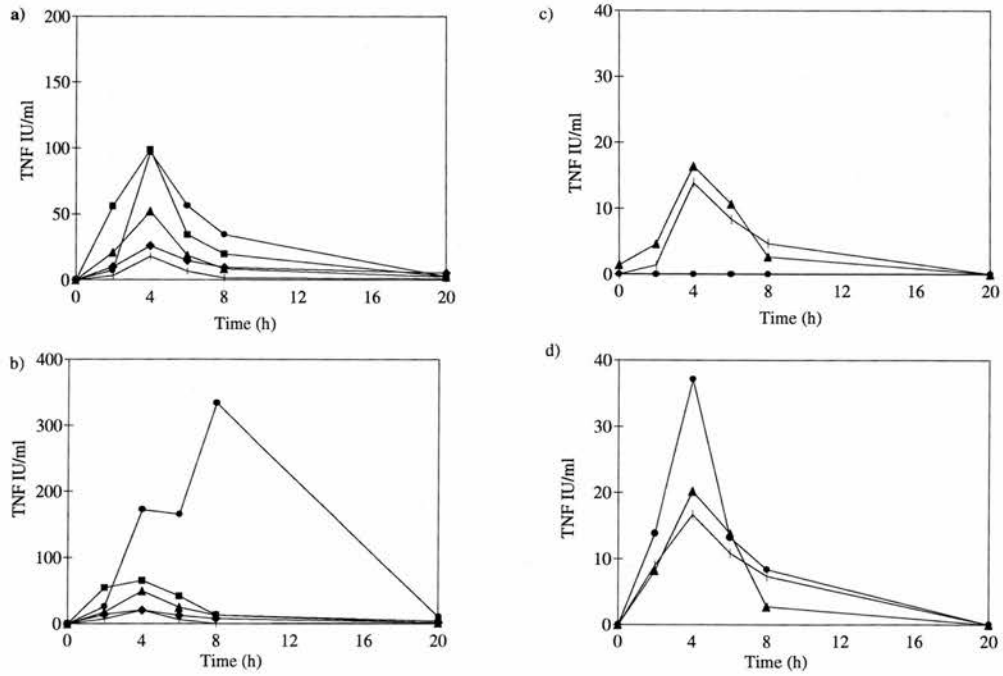

FIG. 4. Time course of TNF production by THP-1 cells without enhancement for CD14 (a), THP-1 cells with enhancement for CD14 (b), peritoneal macrophages from C3H/HeJ mice (c), and peritoneal macrophages from C3H/HeN mice (d) after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ (●), *B. fragilis* NCTC 9343 (▲), *B. caccae* MPRL 1555 (◆), *B. uniformis* MPRL 1721 (■), and *B. vulgatus* MPRL 1985 (|). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on *y* axes.

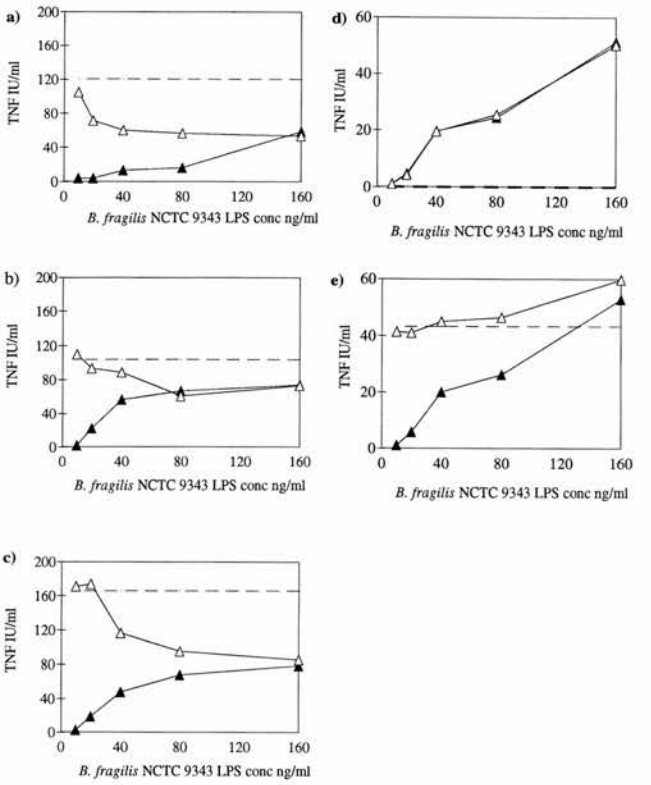

FIG. 5. TNF production after LPS stimulation for 4 h by human mononuclear leukocytes (a), THP-1 cells without enhancement for CD14 (b), THP-1 cells with enhancement for CD14 (c), peritoneal macrophages from C3H/HeJ mice (d), and peritoneal macrophages from C3H/HeN mice (e) after stimulation with various amounts of LPS from *B. fragilis* NCTC 9343 (▲) and various amounts of *B. fragilis* NCTC 9343 LPS together with a constant amount of *E. coli* O18K$^-$ (40 ng/ml) (△). – – –, maximum amount of TNF produced by *E. coli* O18K$^-$ alone. Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on *y* axes.

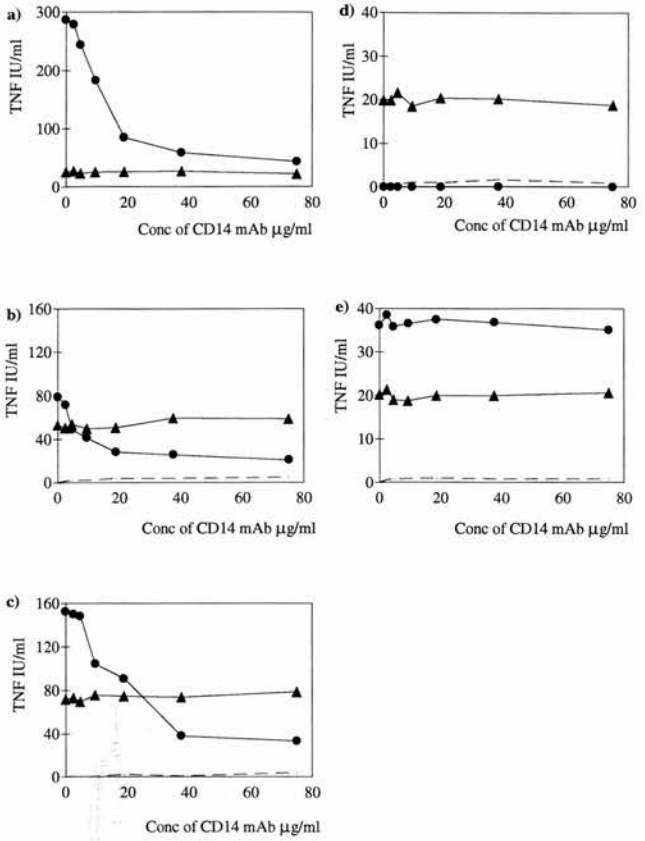

FIG. 6. Inhibition of TNF production after 4 h by a CD14 MAb from human mononuclear leukocytes (a), THP-1 cells without enhancement for CD14 (b), THP-1 cells with enhancement for CD14 (c), peritoneal macrophages from C3H/HeJ mice (d), and peritoneal macrophages from C3H/HeN mice (e) after stimulation with LPS (50 ng/ml) from *E. coli* O18K$^-$ (●), *B. fragilis* NCTC 9343 (▲), and CD14 MAb alone (– – –). Each point is the mean of duplicate experiments performed with the same cell preparations. Note different scales on *y* axes.

TNF production from *E. coli* O18K$^-$ LPS when LPS from *B. fragilis* NCTC 9343 was present in excess. This trend was also seen for *B. vulgatus* MPRL 1985 and *B. caccae* MPRL 1555 (data not shown). For peritoneal macrophages from C3H/HeJ mice, levels of TNF production for *B. fragilis* NCTC 9343 LPS alone and *B. fragilis* NCTC 9343 LPS together with *E. coli* O18K$^-$ LPS were identical: *E. coli* O18K$^-$ LPS alone does not cause TNF stimulation (Fig. 5d). The trend seen in the other cell populations was not clear for peritoneal macrophages from C3H/HeN mice, as the amount of TNF produced by *E. coli* O18K$^-$ LPS alone was lower than that produced by the highest concentration of *B. fragilis* NCTC 9343 LPS (Fig. 5e).

Inhibition of TNF production by an anti-CD14 MAb. The effect of a CD14 MAb in inhibiting TNF production was examined. In all cell populations tested, the presence of a CD14 MAb did not affect the production of TNF stimulated by *B. fragilis* NCTC 9343 LPS (Fig. 6). However, the production of TNF stimulated by *E. coli* O18K$^-$ was inhibited as the concentration of CD14 MAb increased (apart from peritoneal macrophages from C3H/HeJ and C3H/HeN mice). This inhibition of TNF production was more marked in human mononuclear leukocytes and THP-1 cells with enhancement for CD14 (Fig. 6a and c). In the presence of high levels of the CD14 MAb, the second peak of TNF production, after stimulation with *E. coli* O18K$^-$ LPS seen in human mononuclear leukocytes and THP-1 cells with enhancement for CD14 (Fig. 3a and b and Fig. 4b), was absent (data not shown). The CD14 MAb had no effect on the kinetics of *Bacteroides* LPS-induced TNF release (data not shown).

DISCUSSION

Bacteroides LPS has been disregarded by many authors as being much less endotoxic than enterobacterial LPS (14, 32), but in a recent study in our laboratory (5), we showed that the biological activity of *Bacteroides* LPS was dependent on the extraction method used, with the phenol-water method (33) producing LPS with the highest biological activity and the phenol-chloroform-petroleum (8) and the Triton-Mg^{2+} methods (31) producing LPS with low biological activities, in keeping with previous studies. Which extracted LPS type, if any, reflects that in vivo remains to be defined.

The term "*Bacteroides* LPS" is used throughout the study for the aqueous phenol-extracted material. It must be stressed that the "LPS" is not a single molecular species, even of heterogeneous chain length, but is much more likely to be a complex of surface LPSs both rough and smooth, as well as possibly lipid-linked capsular polysaccharides (CP). In SDS-PAGE, the *Bacteroides* LPS appears as a series of several different molecules of high and low molecular mass (Fig. 2 and reference 5). To add to the complexity, some of the molecules can be detected

only by immunostaining, as they are not sensitive to staining by silver (1). The comparison of activity with *E. coli* LPS on a weight-for-weight basis is preferred to a comparison on a molar basis because of the complexity of the different molecular species. In a previous study (5), we used KDO concentration as a basis of comparison and found the same magnitude of differences between *E. coli* and *Bacteroides* spp. Also, when we consider the relative endotoxicity on a bacterium-for-bacterium basis, weight for weight of phenol-extracted LPS is probably more relevant.

From the work of Patrick (23) and Patrick and Reid (24), we know that a pure laboratory culture of *B. fragilis* exists as a collection of subpopulations expressing a range of surface antigens. These subpopulations can be separated on density gradients, and the different antigens can be identified with a series of MAbs (15). The CP of *B. fragilis* has been seen to consist of at least two different molecules (23, 29, 30). An intriguing observation was made several years ago by Onderdonk and colleagues (21), i.e., that the CP of *B. fragilis* could induce sterile abscesses. This issue has recently been readdressed (29), and Cross (4) has suggested that CP may induce proinflammatory mediators such as interleukin-1. We have previously reported that some preparations of CP are contaminated with high-molecular-mass LPS (25). In 1984, Williamson and colleagues (34) recognized that the polysaccharide and lipid A fractions of *B. fragilis* LPS (prepared by aqueous phenol extraction) had different effects on spleen cells of C3H/HeJ mice: the mitogenic response was only to the polysaccharide fraction via macrophages.

Bacteroides LPS is capable of inducing mononuclear cells to produce TNF. This seems to be independent of expression of CD14, the 55-kDa glycosyl phosphatidylinositol-linked protein expressed on the surface of monocytes, macrophages, and polymorphonuclear leukocytes (11), which has been found to serve as a specific binding site for complexes of LPS and LPS-binding protein in serum (16, 35, 36). Although the functional role of CD14 has yet to be fully defined, it plays a very important role in LPS activation events.

In cell populations lacking CD14 (THP-1 cells without enhancement for CD14) and in peritoneal macrophages from C3H/HeN mice, *E. coli* O18K$^-$ LPS could induce only one peak of TNF release at 4 h, compared with peaks at 4 and 8 h in CD14-rich populations (human mononuclear leukocytes and THP-1 cells with enhancement for CD14) (Fig. 3 and 4). The peak of TNF release at 8 h was inhibited by high concentrations of the anti-CD14 MAb (data not shown). *Bacteroides* LPS could induce only one peak of TNF release at 4 h regardless of the cell population, suggesting that it stimulates cells to produce TNF via a pathway independent of CD14 and that the periodic response may be related to CD14. The fact that TNF release stimulated by *Bacteroides* LPS could not be inhibited by an anti-CD14 MAb (compared with *E. coli* LPS, which could be inhibited) is further evidence to support this concept. Of note is that only in human cell populations rich in CD14 is there a significant difference in TNF-inducing capability by LPS from *Bacteroides* species and *E. coli.*

In a recent report by Lynn et al. (17), CD14 and serum were shown not to be absolutely necessary for the activation of mononuclear phagocytes by bacterial LPS. They suggested that a CD14-independent pathway may be of importance in local sites of infection where the concentration of LPS may be high, for example, fecal soiling of the peritoneum. This fact is interesting when we consider our results obtained for peritoneal macrophages from C3H/HeJ and C3H/HeN mice. Here, TNF release after stimulation with both *Bacteroides* and *E. coli* LPSs appears to occur through a CD14-independent pathway: only a single-peak response was seen for both LPSs (Fig. 4), and a CD14 MAb had no effect on TNF release after stimulation with either LPS (Fig. 6). The lack of effect by the CD14 MAb on the TNF response to the *E. coli* LPS by the C3H/HeN macrophages could suggest that the MAb does not recognize the mouse CD14 analog. This has recently been confirmed by flow cytometry (data not shown). Peritoneal macrophages may differ in the ability to respond to LPS compared with circulating macrophages. It has been reported that different macrophage populations differ in their TNF responses to LPS: alveolar macrophages, for example, produce a much higher level of TNF with a faster response than Kupffer cells (3).

In peritoneal macrophages from C3H/HeJ mice, the *B. fragilis* LPS stimulates a low level of TNF production whereas *E. coli* LPS stimulates no TNF production. It is well documented that peritoneal macrophages from C3H/HeJ mice are unable to respond to LPS from members of the family *Enterobacteriaceae* because of a genetic deficiency (27). However, a pathway open to *B. fragilis* LPS is capable of stimulating TNF production. Which component of the *B. fragilis* LPS complex induces this effect is not yet known. Fujiwara et al. (7) demonstrated that LPS extracted by aqueous phenol from oral *Bacteroides* species (now reclassified as *Porphyromonas* and *Prevotella* species) were highly mitogenic to spleen cells from C3H/HeJ mice. Other *Bacteroides* spp. have yet to be investigated.

An excess of *Bacteroides* LPS blocked the effects of *E. coli* O18K$^-$ LPS on human mononuclear leukocytes and THP-1 cells (with and without enhancement for CD14) (Fig. 5). Whether this was due only to the configuration of LPS in solution or due to an effect at the cellular level is not known. A well-documented synergistic relationship exists between *E. coli* and *B. fragilis*, whereby *B. fragilis* inhibits the phagocytic killing of *E. coli* (20, 26). Magnuson et al. also found that *B. fragilis* NCTC 9343 LPS inhibits *E. coli* LPS-induced human endothelial cell adhesiveness for neutrophils (18). However, in that study, it was not found that *B. fragilis* LPS inhibited TNF production, but the *B. fragilis* LPS was extracted sequentially by the phenol-water method followed by the phenol-chloroform-petroleum method. This may have some bearing on the results found.

Our observations may be of some importance when we consider that *Bacteroides* species outnumber *E. coli* in the gut by at least 1,000-fold. It is not yet known if the situation observed in vitro will occur in vivo. However, it may be significant in terms of immunotherapy if bacteria or their products translocate from the gut into the bloodstream. If *Bacteroides* LPS is the major inducer of cytokine responses, we should be targeting them with antibody therapy instead of *E. coli* (1a). However, if the inhibition of the *E. coli* LPS response occurs in vivo, then perhaps the *Bacteroides* LPS serves a protective role.

There are still several questions that remain to be answered. (i) Why are there several peaks of TNF production after stimulation with *E. coli* LPS in CD14-rich cell populations? What causes the troughs in the periodic TNF response? We acknowledge that there may be heterogeneity in the cell population or different stages present in the cell cycle which may cause this response. Also, TNF may be present during the trough but in a bound form which would not be detectable in our bioassay. The possibility that TNF is neutralized during the trough by soluble TNF receptor appears unlikely (unpublished observations). (ii) What is the activation pathway which is independent of CD14, and how does *Bacteroides* LPS stimulate peritoneal macrophages from C3H/HeJ mice to produce TNF? (iii) How does an excess of *Bacteroides* LPS mask the effects of *E. coli* LPS? There is still therefore much to be learned about *Bacteroides* LPS and monocyte/macrophage activation pathways.

In conclusion, the *Bacteroides* LPS complex activates mononuclear cells by an unknown route to produce TNF independently of CD14, and it can mask the effects of *E. coli* LPS. The role of *Bacteroides* LPS in shock remains to be defined, but the results of our previous and current studies indicate that *Bacteroides* LPS may play an important and previously overlooked role in endotoxic shock. *B. fragilis* has a history of debate and some confusion as to the structure-function relationships of its polysaccharide surface structures (14, 25), and it is perhaps too early at this stage to suggest that individual molecules can induce specific mediators. However, the recent work of Tzianabos and colleagues has begun to resolve some of the complexity by identifying two distinct polysaccharides in the CP complex of *B. fragilis* 9343 (30) and proposing a mechanism for the formation of abscesses (29). However, until we can finally fractionate, or clone, the various other constituent molecules, little further progress can be made in determining their individual and combined functions in pathogenesis. This is the important next step.

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Biological Activity of *Bacteroides* Lipopolysaccharide—Reappraisal

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Lipopolysaccharide (LPS) was extracted from six species of *Bacteroides* by the phenol/water, petroleum/chloroform/phenol, and Triton/magnesium methods. Yields and chemical analysis demonstrated that the products were different. Biological activity (endotoxicity) was assessed by the limulus amebocyte lysate (LAL) assay, induction of tumor necrosis factor (TNF) from human mononuclear leukocytes, and a lethality model with galactosamine-sensitized mice. Results showed that endotoxicity varied greatly depending on the species and the extraction method. LPS prepared by the phenol/water method was most endotoxic and that from *Bacteroides fragilis* had the greatest activity. Compared with *Escherichia coli* LPS, the phenol/water extract of *B. fragilis* was sevenfold more active in the LAL assay and marginally less active (five- to seven-fold) in the bioassay for TNF induction. However, when *B. fragilis* LPS was added to *E. coli* LPS, the induction of TNF was inhibited. In the mouse model, *B. fragilis* LPS was 5,000-fold less toxic. If the gastrointestinal tract is the source of the endotoxin in patients with systemic inflammatory response syndrome, then the obligately anaerobic *Bacteroides* species, which outnumber the facultative species such as *E. coli* by 1,000-fold, should not be overlooked.

In the past few years, it has become accepted that much of the systemic endotoxin involved in the pathogenesis of systemic inflammatory response syndrome (SIRS) or sepsis in the severely ill patient originates in the bowel. Bacteria and their products may translocate during episodes of shock-induced gut ischemia [1] to the portal circulation where lipopolysaccharides (LPSs) and possibly other biologically active molecules interact with, inter alia, Kupffer's cells, the macrophages of the liver. Many of the subsequent pathophysiological changes characteristic of SIRS result from the induction of the proinflammatory cytokines, of which TNF-α is one of the most important [2].

In the gastrointestinal tract, the strictly anaerobic *Bacteroides* species outnumber the enterobacteria by 1,000-fold. However, the biological activity of the LPSs of *Bacteroides* is generally considered to be low. In a variety of models, both in vivo and in vitro, it has been reported that the endotoxicity of *Bacteroides* LPSs is 10,000- to 10-fold lower than the activity of LPSs of the members of the family Enterobacteriaceae [3]. Arithmetically, this finding might suggest, even allowing for the much lower biological activity, that within the gastrointestinal tract the pool of potential endotoxin may originate as much from *Bacteroides* species as from *Escherichia coli* and other facultative gram-negative bacteria on a weight-for-weight basis.

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There has been a history of confusion and debate as to the types, structures, and locations of the various surface polysaccharides of *Bacteroides* species, and *Bacteroides fragilis*, the major pathogen of the genus, has been studied in most detail. The following facts have been established: (i) Within a laboratory culture of *B. fragilis*, subpopulations exist that can be separated by density gradient centrifugation with Percoll. Immunogold electron microscopy, immunoblotting, and flow cytometry with monoclonal antibodies have shown that different subpopulations express different saccharide epitopes and, possibly, different entire polysaccharides on their surface and that antigenic variation may occur [4]. (ii) The structure of lipid A of *B. fragilis* LPS has been determined and shown to be monophosphorylated; it has acyl substituents different from those of *E. coli*. The lower endotoxicity of *B. fragilis* LPS is thought to be a direct result of the chemical structure of lipid A [3]. (iii) It has long been recognized that the capsular polysaccharide of *B. fragilis* can induce the formation of abscesses, and recently it has been shown that two different polysaccharides, each carrying a positive and a negative charge, are present within a capsular polysaccharide complex; these polymers, which carry the dual charges, act in tandem to induce the abscess [5]. (iv) The growth environment influences the expression of surface polysaccharides and changes the sensitivity of the bacteria to serum complement [6].

At our laboratory, a major study is under way that is examining the pathogenic potential of different species of *Bacteroides* in several different models and is investigating the possibility that *Bacteroides* species within the gastrointestinal tract represent a vast reservoir of potential endotoxin. The study reported here is a part of the larger study; the aim of this study was the assessment of the biological potential of *Bacteroides* LPSs by determining (1) if different extraction

Table 1. Composition of samples of LPSs.

LPS sample, substance	Concentration (μg/mg dry weight) of substance extracted by indicated method		
	PW	PCP	TM
B. fragilis NCTC 9343			
Carbohydrate	172	91	148
Phosphorus	39	34	49
KDO*	3.8	1.4	2.1
B. vulgatus MPRL 1985			
Carbohydrate	268	67	197
Phosphorus	79	47	38
KDO	2.7	0.9	2.6
B. thetaiotaomicron MPRL 1720			
Carbohydrate	364	44	262
Phosphorus	54	47	80
KDO	. . .	0.4	4.0
B. caccae MPRL 1555			
Carbohydrate	240	30	149
Phosphorus	54	34	25
KDO	. . .	. . .	. . .
B. uniformis MPRL 1721			
Carbohydrate	222	53	173
Phosphorus	102	38	86
KDO	. . .	. . .	. . .
B. ovatus MPRL 1709			
Carbohydrate	281	111	178
Phosphorus	43	40	42
KDO	5.7	1.1	2.4
E. coli O18K⁻			
Carbohydrate	260	ND	ND
Phosphorus	34	ND	ND
KDO	2.4	ND	ND

NOTE. KDO = 3-deoxy-D-manno-2-octulosonic acid; LPS = lipopolysaccharide; MPRL = Department of Medical Microbiology culture collection; NCTC = National Collection of Type Cultures; ND = not done; PCP = petroleum/chloroform/phenol; PW = phenol/water; TM = Triton/magnesium; . . . = below detection limit of assay.

* Concentration measured after dephosphorylation by hydrofluoric acid.

methods influence the chemical composition of the product, (2) the biological activity of the different LPSs, and (3) if different growth media influence the chemical composition and/or the biological activity of LPS.

During this study, we made no attempt to fractionate the individual molecular species of the aqueous phenol–extracted, ultracentrifuged LPS complex, and it may be wrong even to name this material LPS; however, the term is retained by convention. Our intention was to determine the overall properties of the polysaccharide that might represent more accurately the properties of the material released from the bacteria. Subsequently, we hope to be able to relate the properties to individual structures.

LPS was extracted from six *Bacteroides* species (table 1) by the phenol/water (PW) [7], petroleum/chloroform/phenol (PCP) [8], and Triton/magnesium (TM) [9] methods; the PW and PCP methods were performed as previously described [10]. Strict attention was paid to purity throughout the growth, harvesting, and extraction procedures. Pyrogen-free water (Milli-Q; Millipore, Bedford, MA) was used in each procedure. Some protein contamination that was initially present in the PCP and TM extracts was removed by treatment with proteinase K followed by ultracentrifugation and washing. The PW extract treated with proteinase K served as a control and showed that the proteinase K treatment itself was not responsible for any biological activity. The PW, PCP, and TM methods were chosen to produce predominantly smooth, high-molecular-weight material; predominantly rough, low-molecular-weight material; and unselected material by an extraction method much milder than the PW or PCP method, respectively.

Each strain was cultured in a 15-L batch in an LH fermenter (L. H. Engineering, Stoke Poges, UK), and after harvesting and freeze-drying, each batch was divided into three

Table 2. Results of LAL assay of LPS and induction of TNF-α by LPS.

LPS sample, activity	No. of units per indicated extraction method		
	PW	PCP	TM
B. fragilis NCTC 9343			
LAL assay*	228	0.6	72
TNF-α induction†	19	2.5	0.5
B. vulgatus MPRL 1985			
LAL assay	16	0.4	11
TNF-α induction	2.6	0.2	0.4
B. thetaiotaomicron MPRL 1720			
LAL assay	4	0.8	17
TNF-α induction	6.4	0.03	0.2
B. caccae MPRL 1555			
LAL assay	235	0.1	51
TNF-α induction	14	0.2	2.5
B. uniformis MPRL 1721			
LAL assay	14	21	42
TNF-α induction	10	4.8	11
B. ovatus MPRL 1709			
LAL assay	4.3	0.6	46
TNF-α indcution	9	1.4	1.3
E. coli O18K⁻			
LAL assay	32	ND	ND
TNF-α induction	99	ND	ND

NOTE. EU = endotoxin units; IU = international units; LAL = limulus amebocyte lysate; LPS = lipopolysaccharide; MPRL = Department of Medical Microbiology culture collection; NCTC = National Collection of Type Cultures; ND = not done; PCP = petroleum/chloroform/phenol; PW = phenol/water; TM = triton/magnesium.

* Activity determined by LAL assay with a kinetic chromogenic endotoxin kit (Coatest Endotoxin, Chromogenix) was measured in EU/5 ng of LPS.

† Activity determined by TNF-α induction via a bioassay with L929 mouse fibroblasts was measured in IU/50 ng of LPS.

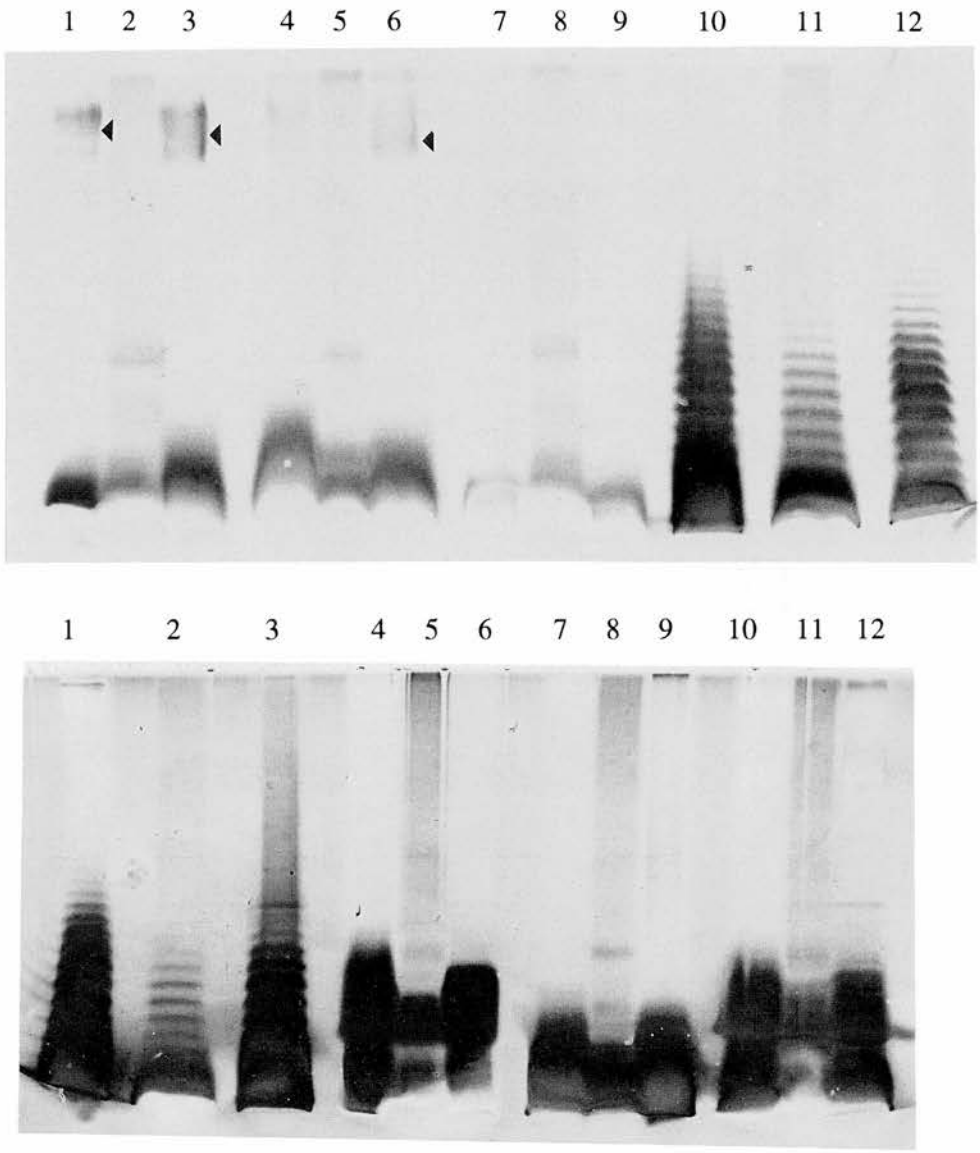

Figure 1. Profiles of silver-stained samples of *Bacteroides* LPSs on polyacrylamide gels. With the exception of the samples in lanes 1–3 of the top figure, all bacteria were cultured in proteose peptone–yeast extract medium. *Top: Lanes 1–3, B. fragilis* NCTC 9343 grown in the medium chemically defined by Van Tassell and Wilkins [11]; *lanes 4–6, B. fragilis* NCTC 9343; *lanes 6–9, B. fragilis* MPRL 1504; and *lanes 10–12, B. vulgatus* MPRL 1985. Arrowhead = position of high-molecular-weight material. *Bottom: Lanes 1–3, B. thetaiotaomicron* MPRL 1720; *lanes 4–6, B. caccae* MPRL 1555; *lanes 7–9, B. uniformis* MPRL 1721; and *lanes 10–12, B. ovatus* MPRL 1709. The first LPS sample in each group of three was extracted by the phenol/water method; the second, by the petroleum/chloroform/phenol method; and the third, by the Triton/magnesium method.

equal samples for extraction. Yields by weight varied considerably; generally, the PCP method demonstrated the highest yield, and the PW method demonstrated the lowest yield. Examples of silver-stained samples of LPSs on polyacrylamide gels are shown in figure 1; these samples were extracted by the different methods from different species. PAGE revealed a variety of different types of profiles. The *B. fragilis* LPSs appeared predominantly rough, but in the PW and TM preparations, high-molecular-weight bands (arrowheads) were apparent. The *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* LPSs had a ladder pattern reminiscent of smooth LPSs of enterobacteria; however, their molecular weights were relatively low, and these preparations lacked the typical high-molecular-weight bands. When immunoblotting with rabbit antiserum to whole bacteria was performed on these preparations, a much more complex picture was generally revealed, including ladder patterns, especially in the higher-molecular-weight regions (E. Allan, S. Riley, and I. R. Poxton, unpublished observations).

Chemical analyses for total concentrations of carbohydrate [12], 3-deoxy-D-manno-2-octulosonic acid (after hydrofluoric acid treatment [10, 13]), and phosphorus [14] were performed. The concentrations of these substances varied greatly depending on the extraction method, with the highest carbohydrate concentrations in the PW extracts and the lowest concentrations in the PCP material (table 1).

Endotoxicity was measured in all samples by the standard limulus amebocyte lysate (LAL) assay with use of a kinetic chromogenic endotoxin kit (Coatest Endotoxin, Chromogenix, Mölndahl, Sweden) (however, the clinical relevance of this assay is being debated), the induction of the pivotal proinflammatory cytokine TNF-α by human mononuclear cells isolated from buffy coat preparations, and a lethality model with D-galactosamine-sensitized mice [15]. A stan-

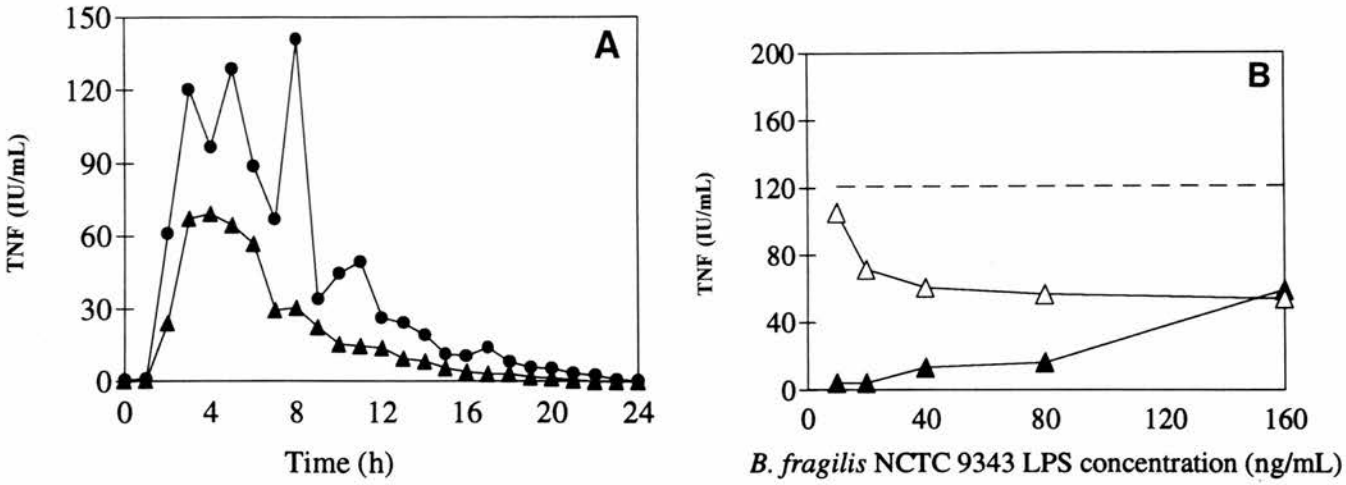

Figure 2. TNF induction by *E. coli* LPS and *B. fragilis* LPS in human mononuclear cells; the induction was determined by a bioassay with L929 mouse fibroblasts. *A*, time course of TNF production (IU/mL) by human mononuclear leukocytes stimulated with LPS (50 ng/mL) from *E. coli* O18K⁻ (●) or *B. fragilis* NCTC 9343 (▲). *B*, TNF production (IU/mL) after 4 hours by human mononuclear leukocytes stimulated with varying amounts of LPS from *B. fragilis* NCTC 9343 (▲) and varying amounts of LPS from *B. fragilis* NCTC 9343 together with a constant amount of LPS from *E. coli* O18K⁻ (40 ng/mL) (△). - - - = maximum amount of TNF induced by LPS from *E. coli* O18K⁻ alone.

dard preparation of the *E. coli* O18K⁻ LPS extracted by the PW method was used as a positive control for comparison in all experiments. The choice of a suitable negative control was considered. However, as so many bacterial components from gram-positive as well as gram-negative organisms have biological activity, it was decided to use the *Bacteroides* LPSs themselves as comparators. All LPSs were prepared in parallel under identical conditions with the same batches of extractants and buffers. Retrospectively, the PW preparations of *B. thetaiotaomicron* or *Bacteroides ovatus* could be considered negative controls because they provided background levels. It was shown that the biological activity was dependent on both the species of origin and the extraction method. Results of the LAL assay and induction of TNF-α are shown in table 2. In the LAL assay, the activity of all *Bacteroides* LPSs extracted by the PCP method was much lower than that of the standard *E. coli* LPS; however, *B. fragilis* and *Bacteroides caccae* LPSs extracted by the PW method were approximately sevenfold more active than *E. coli* LPS, while the activity of *B. thetaiotaomicron* and *B. ovatus* LPSs was much lower. When *B. fragilis* was cultured in the medium chemically defined by Van Tassell and Wilkins [11], the activity was 20-fold more than that of *E. coli* (results not shown).

B. fragilis and *B. caccae* LPSs extracted by the PW method induced TNF-α, but this level of induction was five- to sevenfold lower than that by *E. coli* LPS. In a time-course experiment (figure 2A), the kinetics of TNF induction by *E. coli* LPS followed a complex periodicity compared with *B. fragilis* LPS. An intriguing observation has been made when increasing amounts of *B. fragilis* LPS were added to a standard amount of *E. coli* LPS. Instead of the additive effect expected, *Bacteroides* LPS inhibited the TNF induction by *E. coli* LPS (figure 2B). The basis of TNF induction by *Bacteroides* LPS and this observation of inhibition are the subjects of another study [16].

In the mouse lethality model, the toxicity of all preparations of *Bacteroides* LPS was much lower than that of *E. coli* LPS; most preparations were at least 5,000-fold less toxic.

In the present study and in previously published studies [16, 17], we have shown that the overall chemical composition, appearance on polyacrylamide gels, and biological activity of preparations of *Bacteroides* LPS are very dependent on the extraction method and growth environment. Different *Bacteroides* species produce LPSs with widely different activity, and most importantly, the LPSs of *B. fragilis* and *B. caccae* in particular appear to be much more endotoxic than hitherto realized. Although the clinical relevance is debatable, the LAL assay demonstrates that some preparations of *Bacteroides* LPS are even more active than *E. coli* LPS. The capacity of *Bacteroides* LPS to induce TNF-α in human mononuclear cells is certainly an important observation, and its modulating effect on the biological activity of *E. coli* LPS could have significant consequences in vivo. The lack of lethality in the model of galactosamine-sensitized mice appears contradictory to our thesis that *Bacteroides* LPS is more active than previously thought. However, at present, the induction of low-dose lethality in this mouse model is not well understood, and substances other than TNF and other proinflammatory cytokines may play key roles [18]. From work to be published in detail elsewhere [16], we know that the induction of TNF by macrophages is independent of CD14 expression and that macrophages of LPS-non-responder mice (C3H/HeJ) are as refractile to *Bacteroides* LPS as are those of responder mice (C3H/HeN). Therefore, pathways

must exist that are different from those previously recognized for cytokine induction, and sensitivity to LPS in mouse lethality models cannot be related directly to TNF induction.

If bacterial translocation is an important source of endotoxin in patients with the systemic inflammation characteristic of clinical sepsis, then the anaerobic gram-negative component of the gastrointestinal flora should not be overlooked from the point of view of inducing and/or modulating the response. Current studies are under way in our laboratory that are extending these observations, and we are beginning to develop methods for assessing the amount of exposure to *Bacteroides* LPS in the patient. By analyzing the kinetics of antibodies to *Bacteroides* antigens in the serum from patients with sepsis who are in the intensive care unit, we have shown indirectly that translocation of *Bacteroides* LPS has occurred [19].

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